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VITAMIN C

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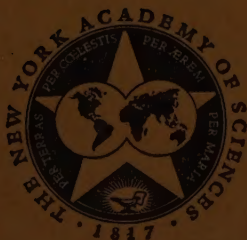
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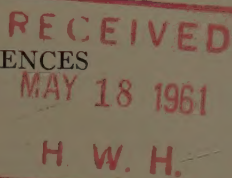
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INTRODUCTORY REMARKS: THE HISTORY AND PRESENT STATUS OF VITAMIN C

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There has been so much progress in research on the origins, end products, and functions of vitamin C in recent years that a monograph of this nature became clearly desirable. Fortunately good reviews of the early literature are available in the record of the Lind Bicentennial Celebration held in Edinburgh, Scotland, in 1953 and in a few additional publications.¹⁻⁹ Probably every reader of this publication is familiar with the record of Lind's famous "controlled" tests: the first studies to be thus designed and interpreted; with the introduction of guinea pigs as test animals by Holst and Frölich in 1912; with the identification of vitamin C as a single crystalline substance in 1932 by King and Waugh and by Szent Gyorgy and Svirbely; with the structured identification and synthesis of the vitamin by several organic chemists in 1933; and with the more recent identification of many functions and the origin of ascorbic acid from glucose and other specific sugars in animals and plants.

Hence the papers in this monograph may deal with very recent progress: and, better still, with questions, theories, and suggestions that are currently in need of exploration.

The records of history as well as recent observations of primitive types of culture establish the point that the human race had to learn many times and under a great variety of circumstances that fresh foods were essential for health and survival. The cost of relearning this relationship, however, without understanding of the nature of the protection afforded by certain foods led to many of the great tragedies in history: voyages by sea and land often failed, long sieges and wars were lost, and millions of people sickened and died because of their failure to understand.

The advent of canned, frozen, and certain types of dehydrated foods and, finally, the synthesis of vitamin C from common sugars have at last freed modern man from such a limitation. Scurvy is no longer a major problem, but instead is confined almost entirely to local individual accidents or carelessness. Nevertheless, the vitamin is so easily lost when foods are not safeguarded that continued diligence is needed to assure an adequate quantity in our food supply.

The remarkable sensitivity of vitamin C to oxidative loss was an important factor in its final identification and in its specific and complete association with scurvy. All the other vitamins in the original A, B, C, and D groups proved to be multiple factors or mixtures. Even in the history of vitamin C there have been many erroneous and confusing reports claiming the existence of other nutrients as part of the causative background of scurvy and the classic symptoms associated with vitamin C deficiency. None of these claims has been sustained, however, by acceptable evidence on careful reinvestigation. The most persistent of these misleading claims has been with reference to a so-called antihemorrhagic factor, represented first by a mixture of materials and later by any one of several substances commonly designated as bioflavo-

noids. These are widely occurring compounds such as catechol, rutin, hesperidin, and others for which vitamin claims are often made primarily to promote their sale. None of these compounds has been demonstrated to have nutrient value in a manner that makes them acceptable for designation as a vitamin^{10,16} or as an essential nutrient. Possibly some readers are not familiar with the literature in this area, such as the papers of Fabianek,¹¹⁻¹⁴ Lee¹⁶ (and elsewhere in this monograph), and many others that are not cited in sales promotion literature.

With respect to the biochemical functions of ascorbic acid, the literature includes an extensive number of reactions that are characteristic of the vitamin when added to plant and animal tissues. Probably these represent functional roles, even though not always specific or dominant. The details of this aspect are far from complete. The dominant oxidizing agents that react rapidly with ascorbic acid are the quinones and related substances with quinonoid structures, the copper proteins, peroxidase systems, cytochrome systems, and metallic ions such as copper, manganese, and iron. The fact that most of these systems are not rigidly specific for the reversible oxidation of ascorbic acid does not mean that they are excluded from its biologic functions. The hydroxylation of other substances by ascorbic acid appears to be an important type of oxidation reaction also, as emphasized by Stewart¹ and others.²

In a similar sense there are numerous reaction systems, particularly involving R-SH compounds, by which dehydroascorbic acid can be reduced to ascorbic acid. The evidence is very limited regarding the extent to which reactions between ascorbic acid and R-SH groups may be dominant in biologic respiratory systems. There is evidence, however, for the formation of very labile, intermediate 1:1 mole compounds between R-SH groups and dehydroascorbic acid (semidehydroascorbate) during the first stage of the apparent 2-electron exchange. This type of intermediate may well prove to be the main reactant in ascorbic acid functions.⁵ Since several of the papers in this monograph deal specifically with the biochemical functions of the vitamin (for example, A. L. Lehninger as well as H. J. Staudinger *et al.*), a detailed review would be out of order at this point.

A brief mention should be made of three relationships that are relatively new. Ettlinger and Lundeen¹⁷ reported that the vitamin functions as a co-enzyme in the hydrolysis of mustard oil glycosides in plants, and Breslow and Lukens¹⁸ have recently made an interesting proposal concerning the mechanism of hydroxylation. Several authors have extended the observations of F. S. Daft and his associates on the sparing action of ascorbic acid on the requirements for certain other vitamins. When large quantities of vitamin C are fed to animals such as the rat, there may be a partial or complete sparing action on the requirement for several vitamins in the B complex. At least two factors seem to be at play in this situation. The first is an effect upon the intestinal microflora in favoring greater synthesis of members of the vitamin B complex; this effect was clarified in recent tests by several authors (for example, by Daft's and B. S. Wostmann's groups) with germfree animals. The second result may be a direct chemical effect in causing greater availability of thiamine, made possible by reducing an inactive dithiamine (a disulfide) form

of the vitamin to its normal active form, as shown by Banhidi¹⁹ and in similar studies by Terroine.²⁰

In addition to recent general progress relative to the vitamin itself,²¹ Chatterjee *et al.* (elsewhere in this monograph) have discovered in India two additional animal species that are subject to scurvy and that lack the specific enzyme that acts on L-gulonolactone to form ascorbic acid (a fruit-eating bat and bird, the red-vented bulbul). This discovery adds a new and very interesting landmark to the history of vitamin C.

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BIOSYNTHESIS OF L-ASCORBIC ACID IN ANIMALS AND PLANTS

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Formation from Hexose Sugars

Feeding experiments. The first convincing evidence that L-ascorbic acid is formed from a hexose was provided by the experiments of Ray,³¹ which showed that with excised pea embryos growing on a synthetic medium, hexose sugars increased the production of the vitamin. It is perhaps significant that with every kind of plant material examined, D-glucose caused the formation of L-ascorbic acid, whereas the other naturally occurring D-aldohexoses have sometimes given negative results. Negative results were obtained with pentose sugars and sugars of L configuration such as L-sorbose, which in view of its close structural similarity to L-ascorbic acid might have been expected to serve as a precursor.^{1,26} In the rat the stimulation by the administration of hypnotic drugs of the excretion of L-ascorbic acid and D-glucuronides suggested that the two might be linked to a common intermediate that might well be D-glucose. In general, however, the evidence for the formation of L-ascorbic acid from hexose sugars in either the plant or the animal was suggestive rather than conclusive. The hexose sugars are not only interconvertible but give rise to a variety of metabolites, among which the substances acting as precursors might be found.

Work with labeled (C¹⁴) sugars. Uniformly labeled glucose fed to "chloretonized" rats¹⁷ gave rise to L-ascorbic acid (in the urine) that was also uniformly labeled. This suggested that the carbon chain of D-glucose was not broken before conversion to L-ascorbic acid or, if so, that the fragments were recombined without any major differential dilution effect. The experiments of Horowitz *et al.*¹¹ and Horowitz and King,¹² in which D-glucose labeled in either C-1 or C-6 was fed to chloretonized rats, giving rise to L-ascorbic acid predominantly labeled in C-6 or C-1 positions respectively, confirmed the suggestion that the molecule was not split. A repetition of these experiments with undrugged rats³ gave essentially similar results although the yield of L-ascorbic acid was less.

In studies on the over-all conversion of D-glucose to L-ascorbic acid in the detached ripening strawberry fruit^{19,23} and in the etiolated, germinating, cress seedling,²⁰ injection or feeding of D-glucose labeled on C-1 gave L-ascorbic with the label chiefly on C-1 (carboxyl) of the L-ascorbic acid. The six-carbon chain of the glucose was conserved, but it was clear that if this evidence were correct the synthesis from D-glucose to L-ascorbic acid must proceed by a pathway that was different from that in the rat. There was no inversion of the carbon chain since the L-ascorbic acid isolated was predominantly labeled on C-1.

This work will be considered in greater detail later in this paper, the important fact being that the synthesis of L-ascorbic acid occurs from D-glucose without breaking the carbon chain.

Mechanism of Conversion

The radiochemical evidence outlined above suggests that a direct conversion of D-glucose to L-ascorbic acid occurs in both the animal and the plant. This means that of the hydroxyl groups on D-glucose, the one on C-5 of the main must be inverted from D to L. This can occur by direct inversion of the groups on C-5 (through a keto group) or by inversion of the whole chain so that C-1 of D-glucose becomes C-6 of the L-ascorbic acid chain; this would make the new C-5 (of L-ascorbic acid) L. There are several possible mechanisms whereby these changes might be realized through biochemically plausible intermediates; a number of these are presented in this paper. Particular attention has been given to suggestions whereby the conversion occurs without inversion of the whole chain (because of the radiochemical evidence obtaining from experiments on plants), and this will be considered first (SCHEME 1).

Included in this scheme are a number of suggestions tentatively advanced by other workers,⁹ namely, that D-fructuronic acid, 3,5-dioxo-D-gluconic acid, 3,5-dioxo-L-gulonic acid, and 5-oxo-L-ascorbic acid are intermediates in a reaction sequence similar to that shown in SCHEME 1. A further complication is that those compounds with P against C-6 may be phosphorylated derivatives in the plant.

On the experimental side we have shown (unpublished data, 1960) that of the various compounds in SCHEME 1 D-glucono- γ -lactone, L-idono- γ -lactone, 3,5-dioxo-D-gluconic methyl ester, and the respective free acids, when fed to pea and cress seedlings and to cabbage leaves, gave no increase in L-ascorbic acid. The same compounds and D-fructuronic acid methyl ester and D-tagaturonic acid methyl ester added to homogenates of soaked peas also gave no L-ascorbic acid. D-Glucurono- γ -lactone gave an increased amount of L-ascorbic acid, but this will be referred to later in connection with another reaction sequence. Of the phosphorylated compounds, the addition of 6-phosphoglucose and 6-phosphogluconate to homogenates of soaked peas gave no L-ascorbic acid.

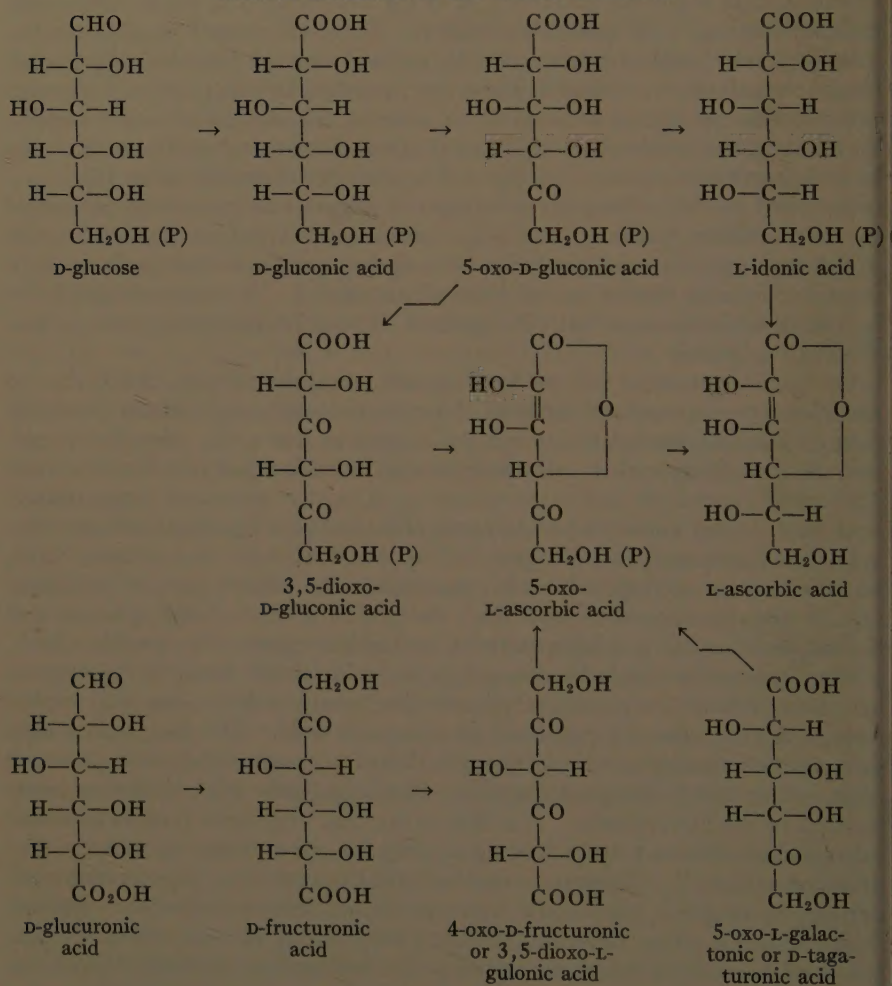
The experiments with 5-oxo-L-galactonic acid methyl ester (D-tagaturonic acid methyl ester) are particularly interesting because L-galactonic acid methyl ester is a very effective precursor of L-ascorbic acid. The fact that a keto group at C-5 entirely prevents the formation of L-ascorbic acid, or an analogue of it, rather upsets the possibility that 5-oxo-L-ascorbic acid can be an intermediate in the biosynthesis. It is safe to say that at present there is no direct experimental evidence from feeding or enzymic experiments to support any case of SCHEME 1. It must be emphasized, however, that if phosphorylated derivatives of these compounds were the actual intermediates, the response might be feeble unless the compounds fed were readily phosphorylated *in vivo*. However, since neither 6-phosphoglucose nor 6-phosphogluconic acid gave any response in a homogenate of soaked peas in which D-galacturonic methyl ester is readily converted to L-ascorbic acid, this possibility does not seem likely. The second scheme for the transformation of D-glucose to L-ascorbic acid involves the inversion of the whole molecule (SCHEME 2).

No experimental evidence has been obtained to support SCHEME 2. None of the intermediates fed to plants (cress seedlings⁸ or parsley leaves¹) gave any increase in L-ascorbic acid. In the rat tracer experiments with L-sorbose-6-C¹⁴,

Burns *et al.*² showed that incorporation of the C¹⁴ occurred only after fragmentation of the carbon chain; this indicated that L-sorbose was not on the direct route from D-glucose to L-ascorbic acid. Injection of labeled 2-oxo-L-gulonic

SCHEME 1

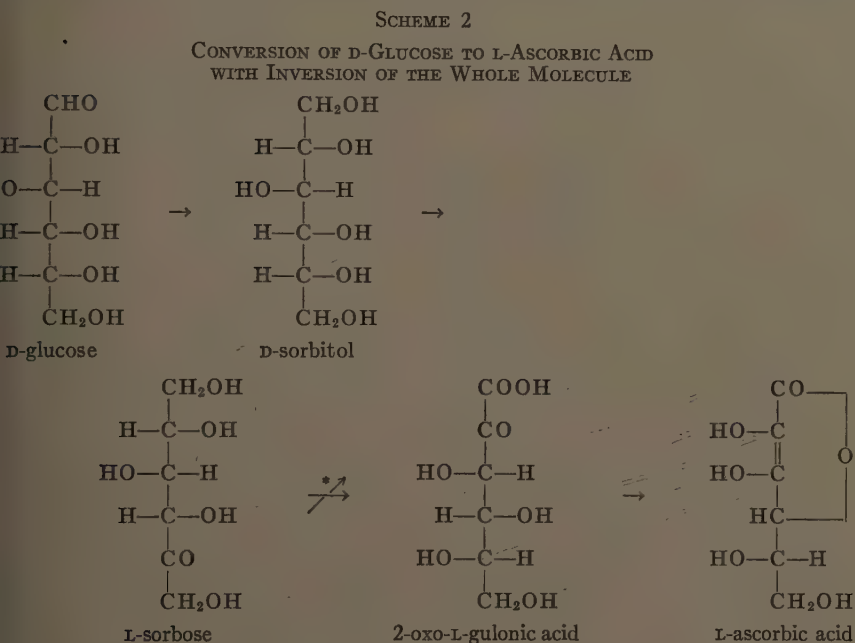
CONVERSION OF D-GLUCOSE TO L-ASCORBIC ACID
WITHOUT INVERSION OF THE WHOLE MOLECULE



acid into rats⁴ also gave negative results. The results with 2-oxo-L-gulonic acid do not exclude the possibility that derivatives of the free acid might behave differently. The methyl ester is converted into L-ascorbic acid in buffer solutions at pH 7.0 without the aid of catalysts³⁴ and has antiscorbutic activity when given to guinea pigs.³² Nevertheless the addition to 2-oxo-L-gulonic

acid, or its methyl ester, of enzyme preparations (from soaked peas and from rat liver), known to be active catalysts for the formation of the vitamin from certain sugar acid lactones, does not cause any significant amount of the vitamin to be formed in the case of the acid, or to accelerate the formation of the vitamin in the case of the methyl ester. This will be referred to again in connection with the suggestion that it is the 2-oxo-L-gulono- γ -lactone that is the immediate precursor of L-ascorbic acid.

A second scheme for the synthesis of L-ascorbic acid from D-glucose that involves inversion of the whole molecule is shown in SCHEME 2 (and a similar



* Configuration as written is inverted.

starting with D-galactose). This is the scheme originally proposed by Isherwood *et al.*¹⁴

A summary of the evidence supporting SCHEME 3 follows.

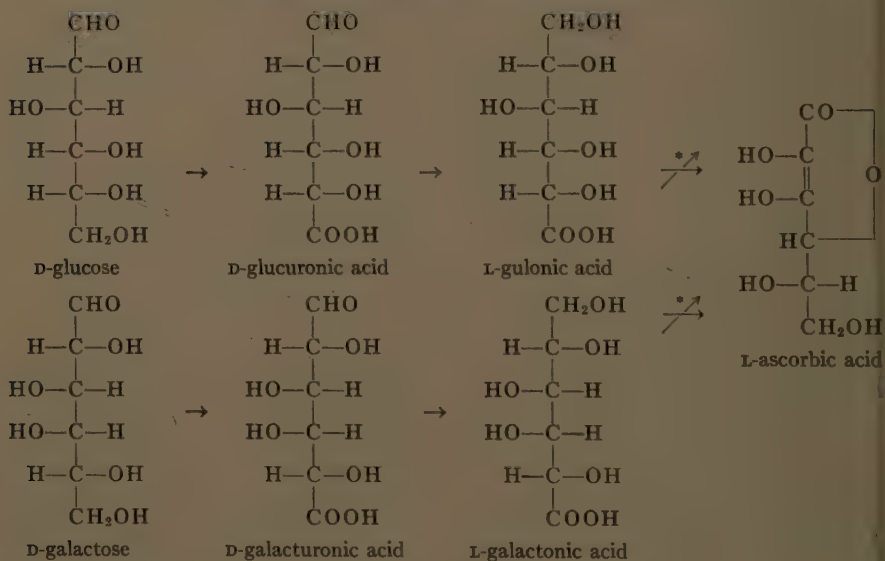
Animal. The scheme was advanced as a result of a study in which a large number of closely related sugar derivatives were injected into rats and also fed to cross seedlings; of these only four were found capable of increasing the excretion of L-ascorbic acid after injection into rats. These were L-gulono- γ -lactone, L-galactono- γ -lactone, and the two corresponding uronic acid derivatives, D-glucurono- γ -lactone and D-galacturonic methyl ester.

The aldolactones gave a bigger response than the uronic acid derivatives, but this was not unexpected in view of the two stages needed for the conversion of the uronic acid derivatives to L-ascorbic acid. SCHEME 3 is also supported by the observations of Longenecker *et al.*²⁵ and Mosbach *et al.*³⁰ that

the stimulation of the formation of D-glucuronic acid by the administration of certain drugs (chloretone) was also accompanied by the increased excretion of L-ascorbic acid. The radiochemical evidence already described and the more recent work of Burns and Evans⁴ in which the feeding of labeled D-glucurono- and L-gulono- γ -lactones to rats resulted in the synthesis of L-ascorbic acid in which the initial distribution of C¹⁴ was retained in the appropriate position on the carbon chain (according to SCHEME 3) further emphasizes the correctness of the hypothesis.

The results of experiments in the intact animal have now been supplemented with work on homogenates of animal tissue and on partially purified enzyme

SCHEME 3

CONVERSION OF D-GLUCOSE AND D-GALACTOSE TO L-ASCORBIC ACID
WITH INVERSION OF THE WHOLE MOLECULE

* Configuration as written is inverted.

systems. Isherwood¹³ first described the formation of L-ascorbic acid *in vitro* using a particulate (mitochondria) preparation from rat liver on L-gulono- γ -lactone, and this has been extended and confirmed since by a number of workers.^{5,6,16} Separation of homogenates into nuclear, mitochondrial, and sub-microscopic particle (microsomes), and soluble fractions showed that the mitochondrial-microsome fractions were alone responsible for the conversion of L-gulono- or L-galactono- γ -lactones into L-ascorbic acid¹⁶ and that the microsomes were more active than the mitochondria. The free acids were not oxidized by the particulate preparations. Treatment of the particulate fraction with deoxycholate gave a "soluble" enzyme that behaved like the original particulate fraction except that the presence of sulfhydryl compounds in the enzyme digest was not necessary. These preparations readily converted

-gulono- γ -lactone into L-ascorbic acid in the presence of O_2 or electron acceptors such as phenazine methosulfate under anaerobic conditions with 2,6-dichlorophenolindophenol as indicator.²⁹ There was no oxidation of L-gulonic or L-galactonic acid. The oxidation of L-gulono- γ -lactone was independent of either DPN or TPN, and these coenzymes were not reduced by the enzymic systems on the addition of the lactone.

It had earlier been found that the addition of 2,4-dinitrophenol to microsomes stimulated the oxidation of L-gulono- γ -lactone by up to 300 per cent, and there was the same stimulation of the "soluble" enzyme. The addition of cyanide, azide, hydroxylamine, and carbon monoxide had little effect on the reaction. This suggests that the terminal oxidase is not sensitive to these reagents. The spectrum of the "soluble" microsomal enzyme was similar to that reported for microsomal cytochrome by Strittmatter and Velick.³³ There was a peak at 413 $m\mu$ in the oxidized state; reduction with sodium dithionite shifted the peak to 523 $m\mu$, with the appearance of two additional peaks at 425 and 556 $m\mu$. However, under anaerobic conditions there was no change in the oxidized absorption spectrum on the addition of L-gulono- γ -lactone; since the extract readily oxidizes this lactone in the absence of added cofactors, it seems evident that microsomal cytochrome does not participate in the oxidative process.

Although the isolated mitochondrial-microsomal fraction and its "soluble" enzyme preparation will not convert L-gulonate to L-ascorbic acid, the whole homogenate (soluble and particulate fractions) will synthesize the vitamin from L-gulonate at a rate that is about one fifth of the rate at which it is synthesized from L-gulono- γ -lactone in the presence of the washed particulate fraction (microsomes and mitochondria). The formation of L-ascorbic acid from the free acid¹⁶ has now been shown to involve an aldonolactonase enzyme³⁵ not present in the particulate fraction as well as an enzyme in the mitochondria and microsomes. It is the combined effect of the soluble aldonolactonase and the microsomal enzyme that converts the free acid through an intermediate L-gulonyl enzyme complex to L-ascorbic acid. No other cofactors are necessary.

An enzyme catalyzing the reduction of the uronic acid derivative to L-gulonic acid in rat liver extracts, similar to that described for plants by Mapson and Isherwood²⁸ has been described by Hassan and Lehninger.¹⁰ This enzyme catalyzed the reduction of D-glucurono- γ -lactone at the expense of TPNH and was located in the soluble nonparticulate fraction of the cell. There seems little doubt that in the case of the animal, *in vivo* and *in vitro* experiments confirm the essential correctness of the scheme suggested by Isherwood *et al.*¹⁴

Plant. The evidence in the case of the plant is not as decisive as in the case of the animal. The main dispute lies in the conflict between the radiochemical experiments of Loewus and the feeding and enzymic studies. In this paper it is proposed to examine the radiochemical evidence first. Loewus has shown that D-glucose labeled at C-1 is converted in the strawberry fruit into L-ascorbic acid labeled also mainly at C-1; he has concluded from this that the vitamin is synthesized by a direct route in which no inversion of the carbon chain occurs. Similar experiments with D-glucose labeled at C-6 gave L-ascorbic

acid chiefly labeled at C-6. As an explanation of these results Loewus has suggested that the reactions of the hexose monophosphate shunt may be involved (SCHEME 1), 3-oxo-6-phospho-D-gluconate being the crucial intermediate. This compound would be converted to 3-oxo-6-phospho-L-idonate and would then lose phosphate to be lactonized and enolized to L-ascorbic acid. However, in view of the discrepancy between the animal and plant radiochemical results, these experiments of Loewus are worth examining in more detail; a representative selection of his radiochemical results is given in TABLE 1, particular attention being given to a comparison of the labeling patterns on the L-ascorbic acid and D-glucose isolated at the same time.

TABLE 1
INCORPORATION OF RADIOACTIVE SUGARS INTO L-ASCORBIC ACID AND D-GLUCOSE*

Sugar**	Subject	Compound analyzed	Labeling (%)			
			C ₁ C ₂	C ₃	C ₄ C ₅	C ₆
G 1	Strawberry	Ascorbic acid	74	4	3	19
GA 1	Strawberry	Ascorbic acid	45	8	6	41
G 6	Strawberry	{ Ascorbic acid D-glucose†	24	1	2	73
			10	0.6	2	86
GL 1	Strawberry	Ascorbic acid	2	0	1	97
GA 1	Strawberry	{ D-glucose† Ascorbic acid D-glucose‡	84	3	2	12
			53	5	3	39
			79	4	3	14
G 2	Rat	{ Ascorbic acid D-glucose§	0 11	8	23 100	31
			23 100	13	3 11	3

* Data from papers of Loewus *et al.*,¹⁰⁻²⁴ and Finkel *et al.*⁹

** Key: G 1, D-glucose-1-C¹⁴; GA 1, D-galactose-1-C¹⁴; G 6, D-glucose-6-C¹⁴; GL 1, D-glucurono-γ-lactone-1-C¹⁴; G 2, D-glucose-2-C¹⁴.

† Free glucose.

‡ Glucose from sucrose.

§ Glucose from glycogen.

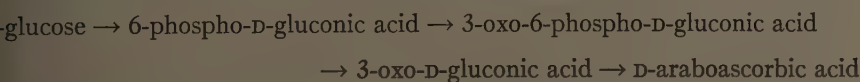
A close examination of the results given in TABLE 1 indicates that in every case, on C-6 of the L-ascorbic acid (if glucose or galactose C-1 is fed) or on C-1 if the sugar is labeled on C-6, the label is 2 to 3 times the label on C-6 of the glucose isolated at the same time from the berry. The increased labeling on C-6 (if glucose or galactose C-1 is fed) is not due to breaking of the carbon chain because the labeling on the other carbon atoms C-3, C-4, and C-5 is no greater than that on the corresponding carbon atoms in the D-glucose isolated at the same time. This is true whether the D-glucose is free in the cell sap or combined in the form of sucrose. This point is emphasized when we compare these results with those obtained in the rat when D-glucose-C-2 was injected (TABLE 1). In this case we find that the label on the L-ascorbic acid corresponds closely with that on the D-glucose (from glycogen) when due allowance has been made for the inversion of the carbon chain (C-1 becomes

-6, C-2 becomes C-5 and C-3 becomes C₄). The results when D-galactose labeled on C-1 was fed to the strawberry are of special interest because the enzymic experiments²⁸ described later indicate that the D-galactose derivatives are much more actively converted to L-ascorbic acid than the corresponding D-glucose derivatives. It is not surprising therefore to find that the radiochemical evidence in the case of D-galactose is even more indefinite than with D-glucose; the labeling on C-6 of the L-ascorbic acid (when D-galactose C-1 was fed) was in extreme cases almost equal to that on C-1. Suggestions that the heavy labeling of C-6 was due to the D-galactose being first fragmented into 3 carbon compounds and then recombined to form a hexose pool from which the precursor of the vitamin was formed, seem unlikely, because the labeling on C₃ and C₄ of the L-ascorbic acid is similar to that on the corresponding carbon atoms in D-glucose isolated at the same time. Comparison of these results with those of an experiment in which L-sorbose-6-C¹⁴ was injected into the rat² shows this fact even more clearly. With L-sorbose in the rat there seems no doubt that fragmentation must occur, and in this case we find that the C¹⁴ is incorporated into the L-ascorbic acid synthesized throughout the C chain (50 per cent between C-1 and C-6 and 50 per cent on remaining C atoms) yet with galactose C-1 in the strawberry, the L-ascorbic formed was still predominantly labeled on C-1 and C-6 (86 and 92 per cent of total), the label on the other C atoms being no greater than that observed in the L-ascorbic acid formed from glucose C-1.

This leads us to consider possible explanations of the results described above. These can be summarized as follows:

(1) There are two pathways in the plant, one involving inversion of the chain as suggested by Isherwood *et al.*¹⁴ and the other not involving inversion. In both pathways the carbon chain is not broken.

(2) Since the experimental evidence for no inversion of the C chain of the glucose depends upon the authenticity of the final sample of L-ascorbic acid isolated, it is perhaps worthwhile to speculate on possible contaminants that could be difficult to separate from the L-ascorbic acid and would simulate L-ascorbic acid in properties. The most likely is D-araboascorbic acid, which could be produced by the following transformations:



The assumption is that either 3-oxo-6-phospho-D-gluconic acid or 3-oxo-D-gluconic acid is naturally present or produced during the rather slow killing¹⁵ of the strawberry fruit by grinding in hot water and that this keto acid is converted to D-araboascorbic acid. The important point is that glucose labeled on C-1 would give D-araboascorbic acid labeled also on C-1. Further work will be necessary to decide whether there are two pathways for the biosynthesis of L-ascorbic acid in the plant.

In contrast to the radiochemical experiments, the feeding *in vivo* experiments and the enzymic *in vitro* experiments definitely support the suggestion that CHEM 3 operates in plants. Feeding D-galacturonic acid methyl ester or galactono-γ-lactone to cress seedlings, mung beans, and peas readily gave

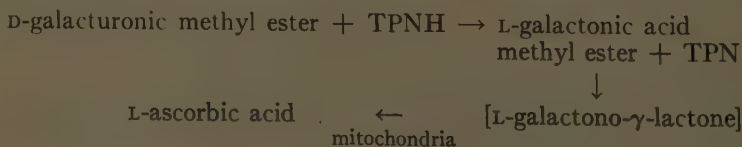
L-ascorbic acid. D-Glucurono- γ -lactone and L-gulono- γ -lactone also gave L-ascorbic acid but in much smaller yield than the corresponding galactose derivatives. It is interesting that very recently (TABLE 1) Finkle *et al.*⁹ have demonstrated by feeding D-glucurono- γ -lactone labeled at C-1 or C-6 to strawberries that the L-ascorbic acid isolated is labeled to the extent of 99 per cent in C-6 or C-1 respectively. This confirms the fact that the reaction uronic acid \rightarrow aldonic acid \rightarrow L-ascorbic acid can operate *in vivo* in plants as suggested by Isherwood *et al.*¹⁴

The results of the feeding experiments have now been supplemented with work on extracts. Mapson *et al.*²⁷ first demonstrated the formation of L-ascorbic acid *in vitro* in extracts from pea seedlings using L-galactono- γ -lactone as substrate. The enzymes responsible were located entirely within the particulate fraction (mitochondria), and their activity depended on the maintenance of the particles in an intact condition. L-Galactono- γ -lactone was rapidly converted to L-ascorbic acid, but L-gulono- γ -lactone only at a rate $\frac{1}{20}$ of that of L-galactono- γ -lactone. L-Galactonic acid was not oxidized. Treatment of the particulate fraction (cauliflower florets) with cold acetone and fractionation of the soluble protein gave a partially purified enzyme that would still oxidize L-galactono- γ -lactone.²⁹ The enzyme required thiol groups for its activity and is a flavoprotein. It is of particular interest that 2,4-dinitrophenol in concentrations known to suppress mitochondrial phosphorylation reactions had absolutely no effect on the rate of conversion of L-galactono- γ -lactone to L-ascorbic acid.

In the case of plant mitochondria, participation of the cytochrome system in the oxidation was demonstrated; the reaction was characteristically inhibited by cyanide, azide, and carbon monoxide in the dark, the latter inhibition being reversed in the light. There was no evidence for the participation of compounds containing "high energy" phosphate bonds; arsenate could replace phosphate as buffer, and ATP inhibited the reaction.

Linked to the above-mentioned enzyme, L-galactono- γ -lactone dehydrogenase, another enzyme was found by Mapson and Isherwood²⁸ in the non-particulate fraction of sucrose-phosphate homogenates of pea seedlings. This enzyme catalyzed a reaction between TPNH and esters of D-galacturonic acid. From chromatographic and kinetic studies, the final product of the reduction appeared to be L-galactono- γ -lactone, although the prior formation of an unstable intermediate was not excluded. The free acid was not reduced, and DPNH could not substitute for TPNH. The γ -lactones of D-glucuronic and D-mannuronic acids were also reduced but only at one-third to one-half the rate of the esters of D-galacturonic acid.

The over-all reactions (suggested as operating in the plant) were as follows although the real intermediates may be, as suggested later, the acyl moieties attached to the enzymes:



Enzymic Oxidation of L-Gulonic Acid in the Animal

It has been mentioned earlier that isolated mitochondria and microsomes from rat liver will convert L-gulono- γ -lactone to L-ascorbic acid but that the mitochondria, microsomes, and the soluble microsomal enzyme will not convert L-gulonate into L-ascorbic acid. However, it was found that an extract of rat liver containing all the tissue elements could convert L-gulonate into L-ascorbic acid. Of various hypotheses, it has been shown that the conversion involves an aldonolactonase enzyme absent in the particulate fraction as well as involving an enzyme in the mitochondria and microsomes. A suggested reaction sequence involves as intermediate an L-gulonyl enzyme complex. The

TABLE 2

ISOLATION OF ALDONOLACTONASE ENZYME FROM RAT AND SHEEP LIVER AND IDENTIFICATION WITH SOLUBLE ENZYMIC FACTOR IN CONVERSION OF L-GULONATE INTO L-ASCORBIC ACID*

Enzyme fraction	Total protein (mg.)	Specific activity (units/mg. of protein)	L-Ascorbic acid synthesized (μ mole/hour)
<i>Rat supernate</i>	8700	5.5	0.23
I	3200	15	0.14
II	1875	25	—
III	230	110	0.12†
IV	66	370	—
V	44	500	0.15; 0.20; 0.25
<i>Sheep supernate</i>	13,000	4	—
II	5000	8.5	—
III	500	90	0.1
IV	260	—	0.15†
V	158	300	0.105, 0.22

* To test each fraction for the soluble enzymic factor, each digest contained reduced glutathione (0.2 mmole), sodium bicarbonate (0.2 mmole), L-gulonate (0.04 mmole), washed microsomes (3 ml. \equiv 3 gm. liver) or a deoxycholate microsomal enzyme (\equiv 3 gm. liver), the aldonolactonase enzyme (\equiv 1000 units), phosphate buffer (0.1 M, pH 7.4 containing 1 mmole Mn^{++} ions) to a total volume of 8 ml. These conditions were such that the amount of aldonolactonase enzyme present was limiting (for details of experiments see Isherwood *et al.*¹⁶).

† Deoxycholate microsomal enzyme.

Decisive evidence in favor of this hypothesis is that a comparison between the activity of the aldonolactonase as a hydrolyzing enzyme and its ability to catalyze the conversion of L-gulonic acid into L-ascorbic acid shows that the two run closely parallel. A summary of the results are given in TABLE 2.

There is no evidence that two separate enzymes were present although the original enzyme was purified 100-fold. A combination of the purified aldonolactonase and the dialyzed soluble microsomal enzyme was equally effective. An aldonolactonase prepared from sheep liver in a manner exactly similar to that described for rat liver was just as effective. The effect of inhibitors on the aldonolactonase and on the complete system for the conversion of L-gulonic acid into L-ascorbic acid also indicated that the enzyme present in the supernate catalyzing the conversion behaved like the aldonolactonase. EDTA (1 mmole) and *p*-chloromercuribenzoate (1 mmole) inhibited both lactonase and synthesis

from the free acid. The effect of the addition of hypnotic drugs on the synthesis of L-ascorbic acid from the lactone, on the one hand, and from the free acid, on the other, was different; the rate with lactone was accelerated, whereas with the free acid it was inhibited as compared with control experiments. This can be explained simply on the inhibition of the aldonolactonase enzyme by the drug.

It is interesting as a comment on the above that as yet we have found no evidence of the presence of an aldonolactonase enzyme in plants (peas).

All workers seem agreed that the inability of the guinea pig, and presumably of all primates, to synthesize L-ascorbic acid is due to the absence of the necessary enzymes for the final stage. We have shown that L-galactono- or L-gulono- γ -lactones when fed or injected into guinea pigs have no antiscorbutic action, and this agrees with the fact that guinea pig microsomes are unable to oxidize either lactone to L-ascorbic acid.⁵

Specificity of the Animal and Plant and Corresponding Enzyme Preparations Oxidizing Aldonic Acid Derivatives

In the case of the plant, the requirements as to the structure of the aldonic acid derivatives are very specific; only those with the same structure for the first four carbon atoms as L-galactono- γ -lactone are readily oxidized. A hydroxyl, either levo or dextro, is apparently required at C-5. Thus in the intact plant (Isherwood and Mapson, using cress, peas, and mung beans; unpublished data, 1960) only L-galactono, D-arabono, D-altrono, and L-fucono- (6-deoxy-L-galactono-) lactones are oxidized at an appreciable rate. L-Gulono- γ -lactone is only slowly converted into L-ascorbic acid in cress. The particulate enzyme preparations show the same specificity; thus L-galactono-, D-altrono-, D-arabono-, and L-fuconolactones are readily converted. L-Gulono- γ -lactone is slowly converted only by enzyme preparations from cress of the plants we examined. D-Tagaturonic methyl ester (5-oxo-L-galactonic methyl ester) is *not* converted (in soaked pea homogenate) to an ascorbic acid analogue; thus a hydroxyl at C-5 is necessary since the configuration of the rest of the molecule is the same as L-galactonic acid. Soluble preparations also show the same specificity although preparations from different sources (cauliflower florets, soaked peas, and fresh garden peas) show widely different activities toward the various analogues of L-galactono- γ -lactone.²⁹

In the case of the animal, there appears to be a difference between *in vivo* and *in vitro* results. With the *in vivo* rat experiments only L-gulono-, L-galactono-, and D-mannonolactones were converted to ascorbic analogues. With the particulate and soluble enzymes L-gulono-, L-galactono-, D-mannono-, D-altrono-, L-fucono-, D-arabono-, D-lyxono-, D-idono-, and D-talono were converted (Isherwood and Mapson; unpublished data, 1960; J. Kanfer *et al.*¹⁸). A summary of our experimental results using various aldonic acid derivatives with enzyme preparations from peas and rats are given in TABLE 3.

There is clearly a big difference between the animal and plant systems, the latter showing a high degree of specificity. The *in vivo* experiments with rats suggest that the whole animal had a higher specificity toward the lactones than the isolated liver mitochondria and microsomes. It is possible that there may

be another enzyme system involved in their transport to the liver that imposes a higher over-all specificity.

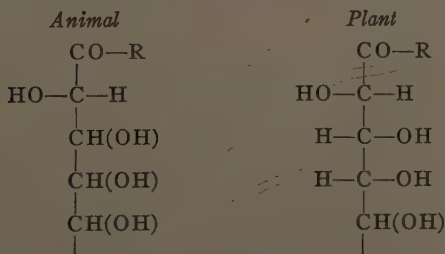
The main features of the specificity requirements are shown in SCHEME 4.

TABLE 3
CONVERSION OF SUGAR ACID DERIVATIVES INTO L-ASCORBIC ACID ANALOGUES BY PARTICULATE ENZYME PREPARATIONS FROM ANIMALS AND PLANTS*

Sugar acid derivative	Ascorbic acid analogue (μ moles/ml. digest/90 min.)	
	Rat enzyme	Pea enzyme
L-Gulono- γ -lactone	1.0	0.0
L-Fucono- γ -lactone	0.55	0.6
D-Arabono- γ -lactone	0.55	0.6
L-Arabono- γ -lactone	0.0	0.0
D-Ribono- γ -lactone	0.0	0.0
D-Lyxono- γ -lactone	1.1	0.0
D-Xylono- γ -lactone	0.0	0.0
D-Idono- γ -lactone	0.7	0.0
D-Talono- γ -lactone	0.45	0.0
L-Galactono- γ -lactone	1.0	1.0
D-Mannono- γ -lactone	0.9	0.0
L-Galactonic acid methyl ester	1.0	—
L-Gulonic acid methyl ester	1.0	—

* With animal preparations, each digest contained reduced glutathione (0.1 mmole), lactone or ester (0.03 mmole), sodium bicarbonate (0.1 mmole), washed microsomes (1 ml. \equiv 3 gm. liver), and phosphate buffer (0.1 M, pH 7.4, 3.5 ml.). With plant preparations, each digest contained lactone or ester (0.03 mmole), mitochondria from soaked peas (\equiv 3 gm. peas), and sucrose-phosphate buffer (0.1 M phosphate, pH 7.4, 0.4 M sucrose, 3.5 ml.). Temperature of incubation 37° C. in air. The ascorbic acid analogue was measured by titration of 1-ml. aliquots after deproteinization with metaphosphoric acid, with 2,6-dichlorophenolindophenol.

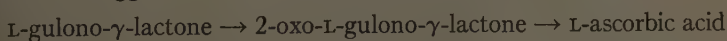
SCHEME 4
ENZYMIC OXIDATION OF ALDONIC ACID DERIVATIVES, MINIMUM STRUCTURAL REQUIREMENT*



* Where R is an ester or lactone group; configuration of CH(OH) can be L or D.

Mechanism of the Conversion of L-Gulono- γ -Lactone to L-Ascorbic Acid

It has been suggested^{7,18} that the reaction sequence is as follows:



The compounds described are supposedly the actual intermediates. The evidence concerning the nature of the actual substrates involved is as follows:

(2) Using a soluble enzyme from rat liver microsomes¹⁸ or the intact microsomes (TABLE 3), it has been shown that only those compounds that have the hydroxyl group on C-2 in the levo configuration (as in L-gulonic, D-mannonic,

(3) In the conversion of L-gulonic acid to L-ascorbic acid by the combined action of the aldonolactonase enzyme and microsomes from rat, a plausible explanation is that the L-gulonyl enzyme complex, formed transitorily from the free acid and the aldonolactonase enzyme, is actually oxidized by the micro-

mal enzyme (possibly with transference of the L-gulonyl moiety to the micro-mal enzyme) rather than a lactone that may δ or γ (and therefore may synthesize either δ or γ ; the aldonolactonase will hydrolyze either δ - or γ -lactones).

(4) Our own experiments (Isherwood and Mapson; unpublished data, 1960) show that concentrated preparations of either rat liver microsomes or pea mitochondria have no accelerating action on the spontaneous conversion (at pH 7) of 2-oxo-L-gulonic methyl ester to L-ascorbic acid.

Based on these facts we suggest the reaction sequence of SCHEME 5.

The real intermediate is not the lactone but the L-gulonyl moiety. We have been struck by the ease with which esters of the various appropriate aldonic acids (as compared to corresponding lactones) are converted to appropriate ascorbic acid analogues (TABLE 3).

A comment on the final enolization stage is perhaps not out of place here. It is well known that the methyl ester of 2-oxo-L-gulonic acid readily goes over to L-ascorbic acid in neutral buffers. A possible mechanism is that it first enolizes; in this form the juxtaposition of the ester group and the hydroxyl on C-4 facilitates the formation of the ring form. The driving force may be the instability of the enolized form of the five-membered lactone (L-ascorbic acid). The same probably happens in the case of the enzymic complex; the rate at which the enol forms is possibly the rate-limiting step and will be similar, whether the ester or the enzyme complex is being considered. The addition of the enzyme to the ester will not therefore noticeably accelerate the conversion to L-ascorbic acid. Treatment of the enzymic complex with metaphosphoric acid (or perhaps spontaneously) will release the 2-oxo-L-gulonic acid that was detected by Chatterjee *et al.*⁷ Extending the idea that it is the acyl moiety that is the active intermediate rather than lactones or esters, we can picture the entire reaction sequence as

glucuronyl-Enzyme I \rightarrow L-gulonyl-Enzyme II

\rightarrow 2-oxo-L-gulonyl-Enzyme III \rightarrow L-ascorbic acid.

The same may be true in the plant starting with D-galacturonic acid methyl ester.

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THE ROLE OF DEHYDRO-L-ASCORBIC ACID AS RESPIRATORY CARRIER IN PLANTS

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Since its discovery by Szent-Györgyi,¹ the theory has often been advanced that ascorbic acid (AA) and its oxidized products form a system that is concerned with the electron transport in the plant cell. The earlier workers emphasized that the vitamin is a universal component of plant cells, is especially abundant in tissues of high metabolic activity, and is rapidly oxidized by at least five enzyme systems extractable from plant tissues, the properties of which have recently been reviewed by Bonner.² Later workers have since established the presence in plant cells of two enzyme systems that are capable of reducing the two oxidized forms of the vitamin, that is, monodehydro-L-ascorbic acid (MDHA) and dehydro-L-ascorbic acid (DHA). The first system involves the participation of diphosphopyridine nucleotide (DPN) and includes a dehydrogenase that transfers hydrogen from oxidizable substrates via DPN to MDHA;³⁻⁵ the second system is one in which the electron transfer is via a triphosphopyridine nucleotide (TPN) and glutathione (GSH) to DHA, and is catalyzed by dehydrogenase enzymes, glutathione reductase, and dehydro-L-ascorbic acid reductase.⁶⁻⁸

Ascorbic acid and its oxidized products thus appear to fulfill many of the requirements necessary before a substance can be deemed to act as an electron carrier.⁹ The object of this paper is to review and describe experiments that have been carried out in attempts to evaluate the role played by DHA as an electron carrier *in vivo*.

Ascorbic acid and respiration. Earlier workers attempted to correlate the respiration of many plant tissues with their AA content. Franke¹⁰ claimed that the increase of respiration of germinating seeds of *Sinapis alba* and the increase in respiration following the cutting of potato tubers ran parallel with an increase in the AA content; Rubin *et al.*¹¹ claimed that infiltration of glucose into the fruit of *Rosa cinnamomea* and *spinosa* led to parallel increases of both respiration and AA content. However, the above correlation was not observed during ripening when the respiration rose but the concentration of AA fell. Other studies, moreover, have shown no correlation between AA and respiration. Mapson and Chen¹² found that the respiration of cress seedlings grown in the presence of certain ammonium salts was not significantly different from seedlings grown in the absence of these salts, although the concentration of AA in the tissue in the former seedlings was only about one third of that in the latter. In more recent experiments with potato tubers (Mapson and Barker, unpublished data), no correlation between the respiratory activity and AA level has been found, tubers of the same variety and stock varying in AA content over a range of 10 to 20 mg./100 gm. tissue, having identical rates of respiration.

This lack of any correlation between the level of AA and respiration does not in itself prove that the vitamin is unconcerned in the respiratory forces.

Synthesis of the vitamin proceeds, in so far as we know, independently of its functioning in respiration; its level in the cell may thus simply reflect the excess formed in synthesis over that used in metabolism. Some plant tissues contain so much AA that it is difficult to believe that it is all being oxidized and reduced in a cyclic process. It seems possible, although no evidence is available on this point, that much of the AA in the cell is in "nonmetabolic pools," only a small fraction of which is operating in any possible respiratory system. That AA is continually being oxidized *in vivo* is supported by our experiments,¹³

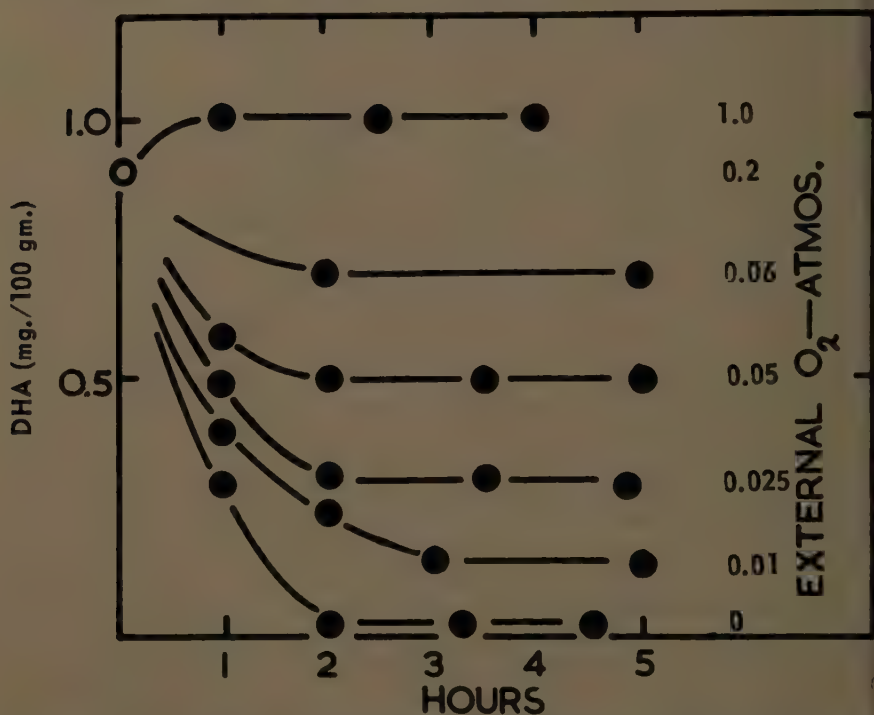


FIGURE 1. The relation between oxygen tension and concentration of DHA in potato tubers.

which show: (1) small but significant amounts of DHA are present in plant tissues under aerobic conditions even when methods of extraction are adopted that, in so far as can be determined, eliminate any oxidation of AA during extraction; and (2) the fall in the AA content of a plant tissue, such as that of potato tubers, stored in air may be prevented if it is stored in nitrogen. This contrast between loss of AA in air, where active synthesis may proceed, and stabilization in nitrogen where synthesis is prevented, underlines the fact that *in vivo* in air a continuous oxidation of AA is proceeding; however, it gives no indication of the magnitude of the process.

Dehydroascorbic acid and oxygen status of the tissue. We have been made aware in our experiments of the greater significance of the level of DHA in

relation to the respiratory activity. The constancy of the respiration of potato tubers of similar age and under similar external conditions was matched by the constancy of the level of DHA in the tissue, whereas the AA content often varied by a factor of 2. Furthermore, on changing the oxygen tension of the atmosphere around the tubers, the level of DHA in the tissues altered in a manner that showed that the new equilibrium level attained was directly related to the oxygen tension in the external atmosphere (FIGURE 1). From a knowledge of the rate of respiration of the tuber and the volume of intracellular space, it may be calculated that conditions in the tuber would be practically anaerobic after about 2 hours in N_2 .¹⁴ The data illustrated in FIGURE 1 show that it took this time for the concentration of DHA to fall to zero.

Such findings have led us to investigate in more detail the relationship between the concentration of DHA and the oxygen status of the tissue. On the assumption that electron transport occurs over DHA *in vivo*, we should expect that the concentration of this constituent would vary directly with the oxygen tension in the tissue; moreover, that the rate at which it is reduced and oxidized could be equivalent to at least a significant percentage of the total oxygen consumption of the extract or respiration of the tissue. If, on the other hand, changes in the level of DHA merely reflect a change in the redox potential of the cell, we should anticipate changes in the concentration of this constituent, but at a rate that would preclude it from bearing any significant proportion of the respiration. In our study of this problem we have examined the changes in AA and DHA in response to alterations in oxygen tension in a number of plant tissues: first, in extracts in order to show unequivocally that enzymic systems oxidizing AA and reducing the oxidized forms of the vitamin were in fact operating; second, in intact tissues to determine the rate of these processes *in vivo*.

Experiments with Extracts

Three tissues have been selected for this study, namely, the maturing pea seed (described hereafter as the green pea), the excized pea embryo obtained from the dried seed after germination, and the potato. Results with pea cotyledons derived from the dried seed during the early stages of germination have already been described in detail by Mapson and Moustafa¹⁵ and are very similar in character to the results we have now obtained with the fresh green peas.

Reduction of Dehydroascorbic Acid and Oxidation of Ascorbic Acid as a Cyclic Process in Extracts of Green Peas

Fresh green peas were selected for this study because we had previously found that extracts of peas maintained their AA in the reduced form under aerobic conditions, a phenomenon that resembles the condition existing *in vivo* but that is not usually found with extracts from plant tissues. The peas (onward, average weight of individual peas 600 mg.) were extracted with 0.4 *M* sucrose, 0.1 *M* phosphate, pH 7.2 to 7.4 buffer solution under conditions that preserve the particulate fractions of the cell;¹⁶ the extract was finally centrifuged at 1500 for 5 min. to remove cell debris. Changes in the AA concentration were then

determined after alteration of the oxygen tension of the solution and/or after the addition of DHA in millimolar concentration; at this concentration the rate of reduction of DHA was found to be maximal. Typical results are illustrated in FIGURE 2A; from this it may be seen that: (1) a change from anaerobic to aerobic conditions leads only to a small initial fall (approximately 15 per cent) in the level of ascorbic acid, and (2) the net rate at which DHA is reduced when added to the extract through which air is being bubbled (0.3 l./min.) is relatively slow compared with its rate of reduction in nitrogen. These results indicated that a system capable of reducing DHA was operative, and that the small

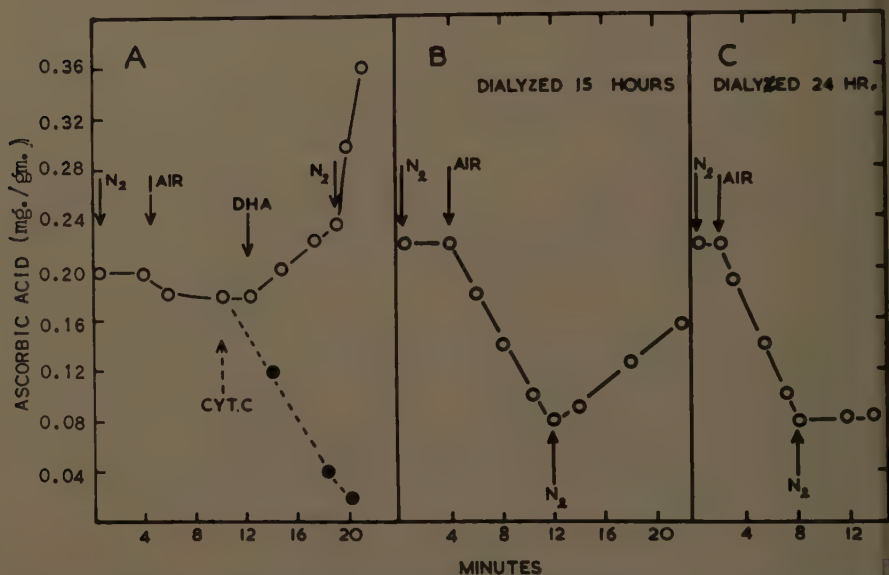


FIGURE 2. The effect of oxygen tension on the level of ascorbic acid and the rate of reduction of DHA in sucrose/phosphate extracts of green peas, freshly prepared and after dialysis for 15 to 24 hours. Extracts prepared from peas by extraction with 0.4 *M* sucrose, 0.1 *M* phosphate, pH 7.4 at 1° C. Additions: cytochrome c 1 μ M, DHA 1 mM, air was bubbled through the solution at a rate of 0.3 l. min.⁻¹. Ordinates: concentration of AA expressed as milligrams per unit weight of fresh peas.

amount of DHA formed on changing from nitrogen to air, combined with a level of ascorbic acid that remained constant for periods of at least 1 hour, could be most simply explained as a result of an equilibrium between systems oxidizing ascorbic acid and those reducing the oxidized forms. Such an explanation might also account for the lower rate of reduction of externally added DHA in air compared to the rate in nitrogen.

This interpretation was supported by the results obtained when the above experiment was repeated on extracts that had been subjected to dialysis at +1° C. against the sucrose phosphate buffer solution. The results illustrated in FIGURE 2B and 2C show that on removal of oxidizable substrates (and/or coenzymes), the efficiency of the reducing system is decreased, as evidenced by the reduced rate of reduction of DHA in nitrogen; this is reflected in the ina-

ility of the extract to maintain its AA in the reduced state under aerobic conditions. A quantitative assessment of these changes is given in TABLE 1. It will be noticed that on extensive dialysis the net rate of oxidation reaches a maximum value that is almost equal to the maximum rate of reduction of DHA by the freshly prepared extract.

These results indicate that in these extracts AA is being oxidized and reduced in a cyclic process. The equilibrium between the process of oxidation and that of reduction was shifted under the following conditions:

(1) When the rate of the oxidative process was increased, for example, by the addition of 10^{-6} *M* cytochrome *c* (FIGURE 2A); this addition also increased the rate of O_2 consumption by 35 to 40 per cent.

(2) When the reducing system was paralyzed by the addition of 0.25 *M* NaI, a salt that we (unpublished data) have recently found to be a potent inhibitor of glutathione reductase (FIGURE 3A).

TABLE 1
RATE OF REDUCTION OF DHA OR OXIDATION OF ASCORBIC ACID IN
SUCROSE/PHOSPHATE EXTRACTS OF FRESH GREEN PEAS

Treatment of homogenate (hours dialyzed)	Rate of reduction of DHA in nitrogen (μ mole/gm./hour)	Net rate of oxidation of AA in air (μ mole/gm./hour)
<i>Experiment 1</i>		
0 (freshly prepared)	13.4	0
15	4.4	7.4
24	1.0	11
<i>Experiment 2</i>		
0 (freshly prepared)	14.0	0
15	4.6	7.5
20	2.9	9.3
30	1.3	10.7

(3) When the oxidase activity was completely inhibited by agents such as sodium diethyldithiocarbamate (DDC) at 10^{-3} *M*; under these conditions the rate of reduction of DHA in air became identical with the rate of reduction in nitrogen (FIGURE 3B).

Condition 3 is of interest in that it suggests that the rate of reduction of DHA in air is unaffected by the presence of other electron acceptors possibly present in the oxidized form in oxygen, but, being completely reduced in nitrogen, are unavailable to compete with DHA.

Relation to oxygen consumption. The full potential reducing capacity of the extract toward DHA in relation to the oxygen consumption of the homogenate and to the respiration of the pea is shown in TABLE 2 from which it may be seen that this relation could account for about 80 per cent of the oxygen consumption of the former and 50 per cent of the respiration of the latter. As we have already seen (TABLE 1), the oxidizing potential is almost equal to the maximum reducing potential, so that the maximum rate at which this system might theoretically operate would be sufficient to mediate a large proportion of the respiration of the pea.

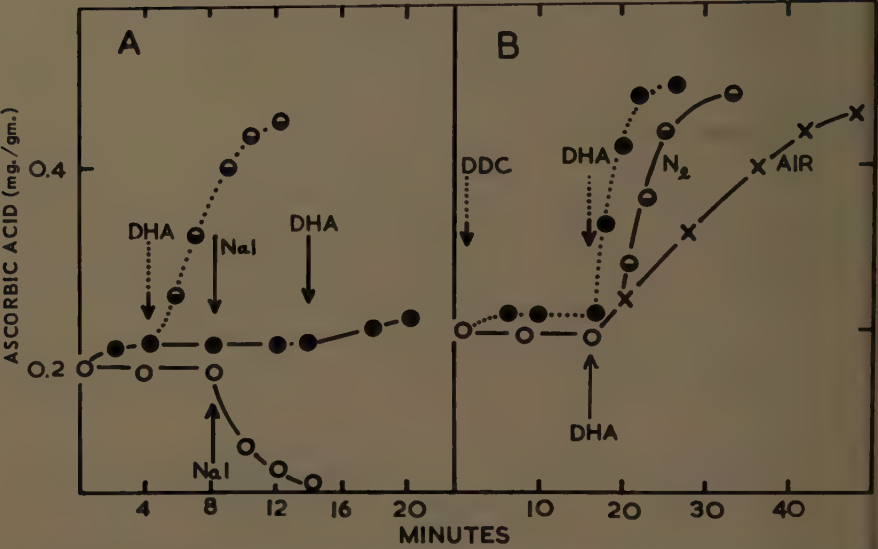


FIGURE 3. (A) Inhibition by sodium iodide of the enzymic system reducing DHA in extracts from green peas. Additions: 0.25 M NaI, 1 mM DHA; ○ In air; ● In nitrogen. (B) The effect of inhibiting ascorbic acid oxidase with sodium diethyldithiocarbamate (DDC) on the rate of reduction of DHA in green pea extracts. Additions: 1 mM DDC, 1 mM DHA; ○ Control; ● With DDC. Ordinates: concentration of AA expressed as milligrams per unit weight of fresh peas.

TABLE 2
RATE OF REDUCTION OF DHA OR OXIDATION OF AA IN EXTRACTS OF
GREEN PEAS IN RELATION TO RESPIRATION OF THE INTACT
TISSUE AND OXYGEN UPTAKE OF THE EXTRACT

Respi- ration (μ mole O_2 /gm./ hour)	Oxygen uptake of homogenate (μ mole O_2 / gm./hour)	Maximum rate of re- duction of DHA in nitrogen* (μ mole O_2 / gm./hour)	Estimated rate of reduction of DHA in extract†			Rate of oxidation of AA in fully dialyzed extract		
			(μ mole O_2 /gm./ hour)	Rate as per cent of res- pira- tion	Rate as per cent O_2 up- take of ho- mogenate	(μ mole O_2 /gm./ hour)	Rate as per cent of res- pira- tion	Rate as per cent O_2 up- take
10.4	7.7	5.4	2.1	20	27	4.9	48	63
12.0	8.0	6.3	2.4	20	30	5.0	42	62
	(Soluble fraction 6.5)	(95% activity in soluble fraction)						
12.9	10.8	7.5	2.9	23	27	5.5	43	51
13.8	11.2	—	—	—	—	6.0	44	53
	(Soluble fraction 8.3)							
11.6	8.5	6.7	2.6	23	31	4.9	42	58
11.0	8.4	7.0	2.7	25	32	4.8	44	57

* Rate of reduction of DHA in nitrogen by extract with DHA at 10^{-3} M.

† Rate calculated on rate of reduction of DHA at a concentration of 10^{-4} M, the concentration found in the extracts in air.

However, the rate at which electrons are transferred to DHA will depend *inter alia* on (1) the actual concentration of DHA and (2) the substrate affinity of the enzyme catalyzing its reduction. Previous work has shown^{6,16} that dehydroascorbic acid reductase, the only known enzyme capable of catalyzing the reduction of DHA, is present in peas. From Yamaguchi and Joslyn's¹⁷ results with the partially purified enzyme, the K_m has been calculated to be of the order of $3 \times 10^{-4} M$. We have determined in our extracts how the rate of reduction of DHA was affected by its concentration. Our results (FIGURE 4) show that half maximal rate of reduction was achieved with a concentration

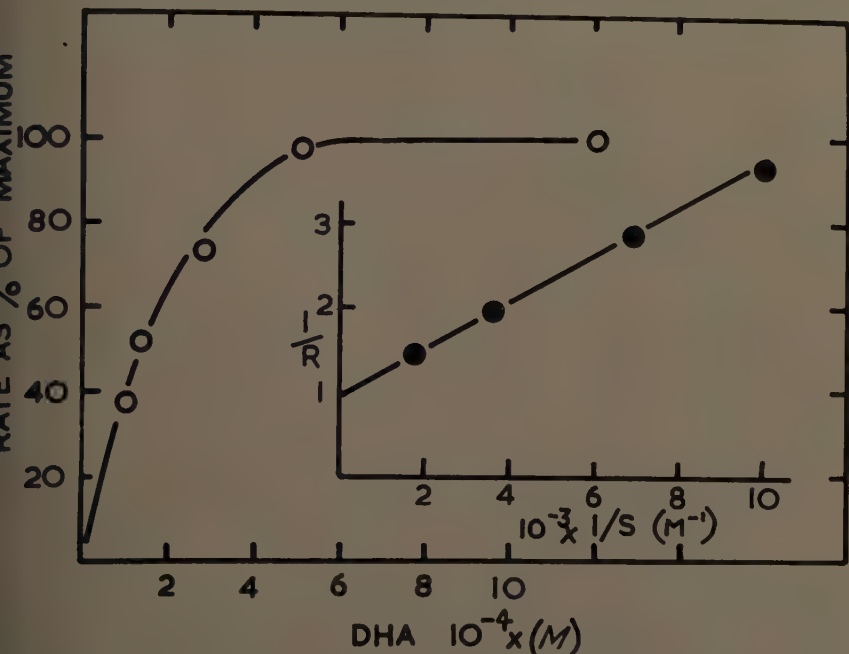


FIGURE 4. Substrate affinity relationship of the enzyme reducing DHA in extracts from green peas.

DHA of $1.5 \times 10^{-4} M$. The concentration of DHA in our extracts under aerobic conditions was of the order of $10^{-4} M$, which shows that its rate of reduction was limited by its concentration. At $10^{-4} M$ the actual rate of reduction of DHA may be estimated as being a little more than one third of the maximum rate. No such limitations apply to the activity of the oxidase. In our extracts the mean concentration of AA was of the order of $10^{-3} M$, at which level the enzyme was saturated with its substrate. With these and the former considerations in mind, it is clear that the ability of the extract to maintain its AA chiefly in the reduced form in air cannot be solely due to a balance between the rate of reduction of DHA and the rate at which AA is oxidized, since our estimates the rate of the former process was only half of the latter. We thus reach the conclusion that other reducing systems are operating, in addition

to those reducing DHA; it seems probably that the system involved is one in which MDHA is the immediate electron acceptor. Since we had no means of measuring this labile constituent, we present this evidence as only suggestive of its functioning in these extracts.

From the data shown in TABLE 2 the electron transport over DHA could account for about 30 per cent of the total oxygen consumption of the homogenate as determined from its estimated rate of reduction by the extract; similarly the participation of the MDHA system could account for a further 25 per cent as adjudged by the difference between the rate of reduction of DHA and the rate of oxidation of AA. The total electron transfer over both these systems

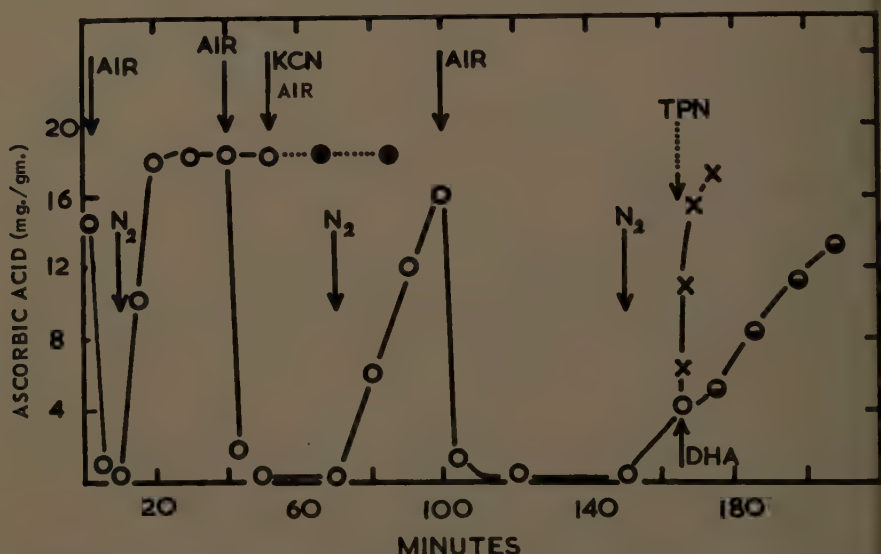


FIGURE 5. The oxidation of ascorbic acid and reduction of DHA with changing oxygen tension in extracts from pea embryos. Embryos extracted with sucrose/phosphate buffer solution as before. Additions: KCN 1 mM, DHA 1 mM, and TPN 0.1 mM. Ordinates: concentration of AA expressed as milligrams per unit weight of fresh peas.

could therefore account for 50 to 55 per cent of the oxygen consumption of the extracts, a result in agreement with the fact that the complete inhibition of ascorbic acid oxidase by DDC (1 mM) decreases the oxygen consumption of the extract by the same extent (53 to 58 per cent).

Extracts from pea embryos. A similar series of experiments has been carried out with sucrose/phosphate extracts prepared from pea embryos separated from their cotyledons 5 to 10 days after germination at 20° C. Qualitatively the same cyclic oxidation of AA and reduction of DHA were observed when the oxygen tension in solution was changed (FIGURE 5); the chief difference was the presence of a much more powerful oxidizing system in these extracts. This was revealed by the fact that to maintain the AA chiefly in the reduced form, the partial pressure of oxygen had to be reduced to 0.019 atm.; at partial pressures above this, the vitamin was rapidly oxidized. There were also other in-

resting differences: the oxidase in the green pea was almost entirely confined to the nonparticulate fraction of the cell (soluble fraction not sedimented at 10,000 g in 30 min.) and was indistinguishable from ascorbic acid oxidase as judged by the criteria of Butt and Halloway¹⁸ (inhibited by DDC, cyanide, and hydroxyquinoline, but not inhibited by EDTA, and with identical rates of reaction between 4 to 40 mM ascorbate), while the mitochondria oxidized AA at an almost negligible rate (5 per cent of the rate of the soluble fraction), which could, however, be increased 20 times by the addition of cytochrome c (10^{-6} M). In contrast, both mitochondrial and soluble fractions from the pea embryo rapidly catalyzed the oxidation of AA, the enzyme in the soluble fraction having the properties of ascorbic acid oxidase, and the enzyme in the mitochondria having the properties of cytochrome oxidase; the oxidative activity was about equally divided.

TABLE 3

RATE OF REDUCTION OF DHA OR OXIDATION OF AA IN SUCROSE/PHOSPHATE EXTRACTS OF PEA EMBRYOS IN RELATION TO THEIR OXYGEN UPTAKE AND TO THE RESPIRATION OF THE INTACT TISSUE

	Respiration (μ mole O ₂ /gm./hour)	Oxygen uptake of homogenate		Maximum rate of reduction of DHA in nitrogen		Net rate of oxidation of AA	
		(μ mole O ₂ /gm./hour)	Rate as per cent of respiration	(μ mole O ₂ /gm./hour)	Rate as per cent of respiration	(μ mole O ₂ /gm./hour)	Rate as per cent of respiration
Day embryo	18	—	—	4.2	23	12	65
Day embryo	14.5	4.6	31	3.3	22	9.1	62
Day embryo	12.1	4.0	33	1.8	15	8.5	69
Day embryo	11.7	3.3	28	1.7	15	—	—

One other noteworthy feature of these extracts was the slow progressive fall in the rate at which added DHA was reduced in nitrogen after successive periods in air. This was found to be due to the oxidative destruction of TPN, because the full activity of the reducing system could be restored by the addition of the coenzyme (FIGURE 5). Incidentally, the fall in the rate of DHA reduction was paralleled by a similar fall in the rate of reduction of added oxidized glutathione (GSSG), which was also restored by the addition of TPN. This is additional confirmatory evidence that the reduction of DHA is dependent on the functioning of the enzyme systems reducing TPN and glutathione reductase. A quantitative analysis of the rates of reduction of DHA and oxidation of AA in pea embryo extracts is given in TABLE 3, which supplements that already published by Mapson and Moustafa.¹⁵ Owing to the activity of the oxidizing enzymes, all the ascorbic acid in air was present in the oxidized form, chiefly as DHA (10^{-3} M). As a consequence the rate of reduction of this constituent was not limited by its concentration but was equal to the maximum rate. Even the net rate of oxidation of AA in these extracts was equivalent to about 60 per cent of the total electron transfer in respiration and was 3 to 4 times greater than the rate of reduction of DHA, which decreased with age of the seedling.

If we gauge the rate of electron transfer over the AA system by the rate of these oxidative reactions, a quite appreciable percentage of the total respiration might be mediated through this channel. It is impossible, however, to draw any conclusions from such data because the conditions in these extracts are so obviously different from the conditions existing *in vivo*. As pointed out by Mapson and Moustafa¹⁵ and confirmed here, the oxygen uptake of these extracts represents only one third or less of the respiration of the intact tissue. Moreover, as we have shown, the extracts fail to maintain their AA under normal aerobic conditions, in contrast to the stabilization of the vitamin in the tissue *in vivo*. Obviously the balance between oxidation and reduction has been materially altered as a result of the process of extraction. It is impossible from these experiments, however, to decide whether the process of reduction has been diminished, or that of oxidation increased, or whether both have been altered. As shown later evidence suggests that reduction has not diminished; that instead the process of oxidation has been accelerated.

Rate of Reduction of Dehydroascorbic Acid and Oxidation of Ascorbic Acid in Green Peas, Pea Embryos, and Potatoes

Peas and pea embryos. I have described experiments in which it has been shown that systems reducing DHA and oxidizing AA are operating in extracts prepared from both green peas and pea embryos. As judged by the estimated rate of reduction of DHA, about 20 to 30 per cent of the oxygen consumption could be mediated via this compound in extracts from green peas and pea embryos (TABLES 2 and 3). No one supposes that the conditions, even in those extracts in which the AA level is stabilized, are identical with those in the intact tissue; it was therefore our aim to determine the rates of these reactions *in vivo*. Therefore we followed the changes in the concentration of DHA when peas, pea embryos, or potatoes were transferred from air to nitrogen or vice versa.

The method of experimentation was twofold: in the first case we studied the rate of reduction of DHA consequent to placing peas or pea embryos in an atmosphere of nitrogen; in the second place we followed the rate of increase in the concentration of DHA in the tissue, which was transferred to air after having been rendered anaerobic by a period in nitrogen. The rate at which DHA was reduced or formed was then computed from an analysis of changes in the DHA levels in short periods of time (15 to 60 sec.). The results of typical experiments with these tissues are illustrated in FIGURE 6. We attribute the slower rate of fall in the first short period after placing the tissue in nitrogen (15 sec. in the case of the green pea; 60 sec. with the embryo) to the presence of oxygen in the intercellular spaces, the full rate of reduction not being attained until this oxygen has been consumed in respiration.

It should be emphasized that for a number of reasons the actual rates observed may be only first approximations to the real rate *in vivo*. Thus with the older pea embryos, as shown by Mapson and Moustafa,¹⁵ the presence of oxygen retards the reduction of DHA by reason of competition from other electron acceptors; the rate of reduction *in vivo* might thus be less than that actually observed. On the other hand, the true rate of reduction of DHA can be measured only after the complete inhibition of the oxidizing systems, which

these experiments was effected by removal of oxygen. When this is completed the DHA concentration has decreased; thus the maximum rate of reaction actually observed will be less than *in vivo*. Similarly the rates of oxidation of AA observed after transfer of the tissue from nitrogen to air are really not rates that are likely to be low estimates since the reducing system will increase in activity as the concentration of DHA increases during oxidation. This latter error has been minimized by calculating rates based on the rate observed only over the first short period of the reaction, when the concentration of DHA had risen by no more than 20 to 30 per cent of the normal level. It is

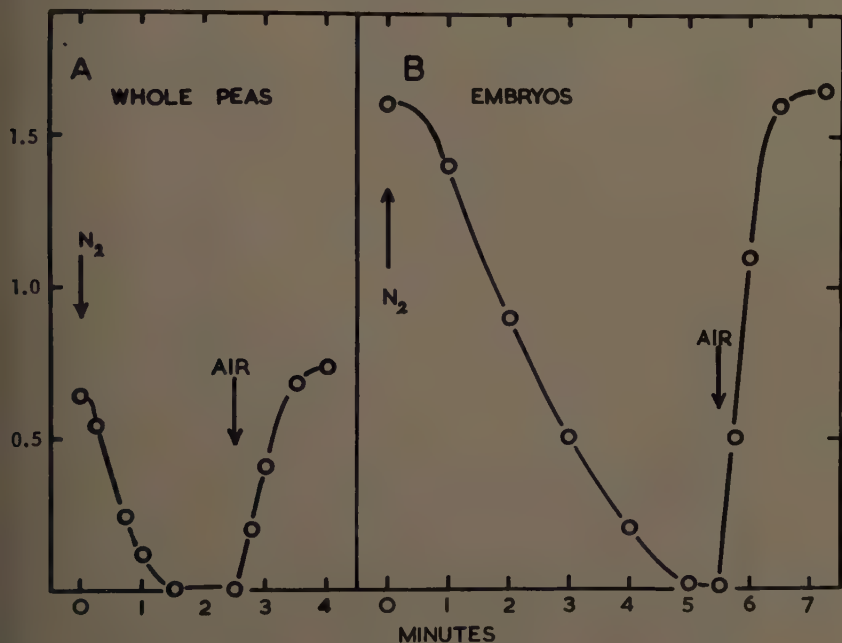


FIGURE 6. Rate of reduction of DHA and oxidation of AA in peas and pea embryos on changing from air to N_2 , and vice versa.

possible to assess precisely the magnitude of these and other possible errors, but it seems unlikely they will materially affect the main conclusions.

Two points are of interest: with green peas the rate of oxidation of AA on passing from nitrogen to air was only about 70 per cent greater than the rate of reduction of DHA on passing from air to nitrogen, whereas with the pea embryo the rate of oxidation of AA was four to five times greater than the rate of reduction. These characteristics were qualitatively similar to the results found with the extracts and afford an explanation for the fact that the level of DHA in the pea embryo in air is 2 to 3 times higher than its concentration in the pea seedling. The results also underline the point made earlier that the disparity between the rate of reduction and oxidation, combined with the fact that *in vivo* the AA is stabilized, suggests that the MDHA enzyme reducing system is operative.

A quantitative assessment of these results (TABLE 4) shows that in the case of the pea, the passage of electrons over DHA can amount to only about 9 per cent of the total electron transfer in respiration. This estimate agrees fairly well with the estimate of the role played by DHA in the oxygen consumption of the extract if we bear in mind that the concentration of DHA in the pea ($0.3 \times 10^{-4} M$) is 2 to 3 times less than that in the extract; as a consequence we should expect its rate of reduction to be 2 to 3 times less than in the extract,

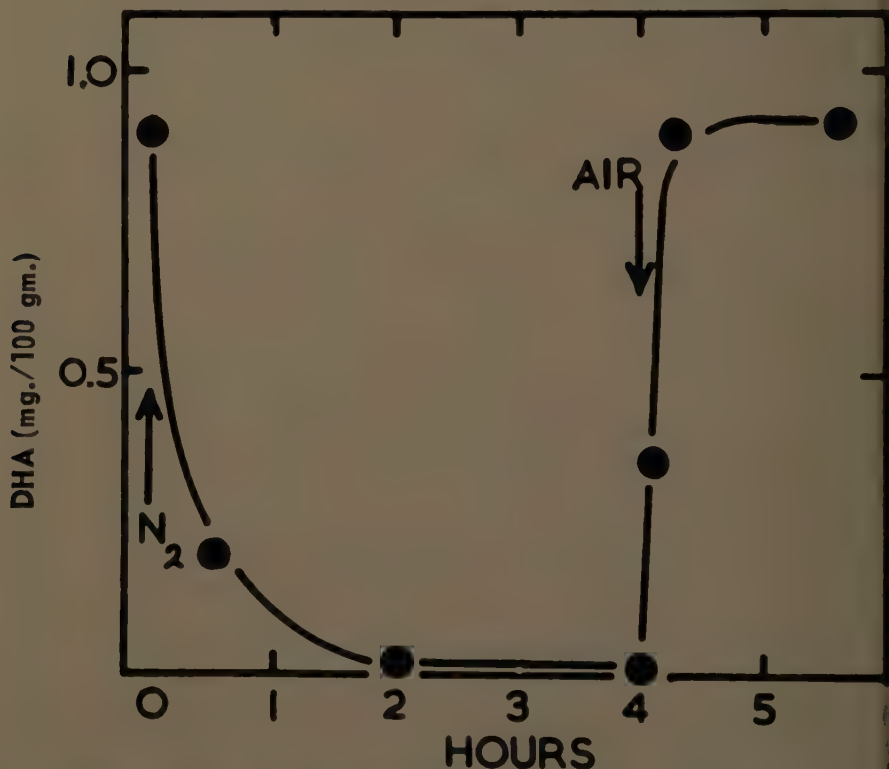


FIGURE 7. Rate of oxidation of ascorbic acid in potato tubers on changing from nitrogen to air.

which in fact it is. Similarly with the pea embryo: the passage of electrons over DHA is less than 10 per cent of the total electron transfer. The slower rate of reduction of DHA in the embryo *in vivo* compared with its rate of reduction in extracts can also be similarly explained on the basis of differing concentrations of DHA (that is, $10^{-4} M$ in embryo and $10^{-3} M$ in extract). On the other hand, there is a discrepancy between the determined rates of oxidation of AA in the extracts compared with the rate of oxidation *in vivo*. With both tissues the rate of oxidation *in vivo* is only about one third that in the extract. Several reasons might be advanced to explain this: (1) alteration of the concentration of enzymes during extraction; (2) inactivation of competitive enzyme

tems; or (3) in the extracts all the AA is available to the enzyme, whereas *in vivo* the concentration of the vitamin at the enzyme surface may not be the same. Whatever the cause, it seems clear that, as a result of the process of fractionation, the activity of the enzymes oxidizing ascorbic acid has been altered to a greater extent than the activity of those enzymes associated with the reduction of DHA.

TABLE 4
RATE OF REDUCTION OF DHA OR OXIDATION OF AA
IN FRESH GREEN PEAS AND PEA EMBRYOS

Respiration (μ mole O_2 /gm./hour)	Rate of reduction of DHA in nitrogen			Rate of oxidation of AA in air		
	(μ mole/ gm./hour)	(μ mole O_2 / gm./hour)	Rate as per cent of respiration	(μ mole/ gm./hour)	(μ mole O_2 / gm./hour)	Rate as per cent of respiration
Green peas						
0.8	1.9	0.95	9	2.8	1.4	14
2.0	2.1	1.05	9	3.5	1.75	15
1.5	2.0	1.0	9	3.3	1.65	14
0.5	1.8	0.9	8.5	2.4	1.2	12
1.5	2.1	1.05	9	3.4	1.7	15
Pea embryos						
4.5 (5 days)	1.9	0.95	6.5	8.2	4.1	28
4.0 (6 days)	1.8	0.90	6.5	7.0	3.5	25
1.7 (8 days)	1.6	0.80	7	6.9	3.5	29
1.8 (8 days)	—	—	—	7.1	3.6	30

TABLE 5
RATE OF REDUCTION OR FORMATION OF DHA IN POTATOES ON CHANGING
FROM AEROBIC TO ANAEROBIC CONDITIONS AND VICE VERSA

Date	Material	Treatment: time under vacuum with N_2 or return to air from N_2 (min.)	DHA reduced or formed		Respira- tion (μ mole O_2 / 100 gm./ hour)	Rate of re- duction or oxidation as per cent respi- ration
			(μ mole/ 100 gm./ hour)	(μ mole O_2 / 100 gm./ hour)		
1.12.59	EE Streatham	Air to N_2 (5)	27	13.5	27	50
		Air to N_2 (5)	28	14	28	50
6.6.60	KE Teneriffe	Air to N_2 (5)	25	12.5	27	46
7.6.60	KE Teneriffe	Air to N_2 (4)	26	13	26	50
		Air to N_2 (4)	28*	14	26	53
4.6.60	KE Teneriffe	Air to N_2 (4)	31	15.5	26	59
		Air to N_2 (4)	24*	12	26	46
5.6.59	KE Teneriffe	N_2 to air (5)	26	13	27	48
10.10.59	KE Teneriffe	N_2 to air (5)	40	20	25	80
2.10.59	KE Teneriffe	N_2 to air (6)	34	17	25	68
3.10.59	KE F.L.	N_2 to air (3)	27	14	23	61
3.10.59	KE F.L.	N_2 to air (6)	30	15	23	65
7.12.59	KE Streatham	N_2 to air (3)	43	21.5	29	74
7.12.59	KE Streatham	N_2 to air (5)	40	20	29	69

Assay by 2,4-dinitrohydrazine method, remainder assay of DHA by indophenol titration or reduction by homocysteine.

Potatoes. Small tubers (average weight, 20 gm.) were used in these experiments not only to minimize diffusion pathways but also to extract the tubers whole, thus avoiding any prior cutting process that would otherwise entail the oxidation of AA and give rise to artificially high DHA values. Because of the relatively slow rate of tuber respiration (about one fiftieth the rate of the pea tissue), we modified the method of determining the rate of reduction of DHA when the tubers were transferred from air to nitrogen. For quick removal of oxygen from the tissue, the tubers were subjected to a vacuum (2 to 5 cm. Hg.) with alternate flushing with nitrogen. This technique was repeated three times, taking a total of 2 to 3 min. to complete. The tubers were then immediately extracted or, if necessary, left for longer periods in nitrogen. In the determination of the rate of oxidation, the tubers were not treated by the vacuum technique but were placed in nitrogen for 1 to 2 hours, rendering them anaerobic. The rate of increase of DHA on return to air was then determined in the tubers.

The results are summarized in TABLE 5. From the data it is apparent that in tuber tissue, in contrast to the pea cotyledon and pea embryo, the rate of reduction of DHA or oxidation of AA represents an appreciable part of the total electron transfer in respiration. A further contrast with the previous results is that the reductive process appears to proceed at a rate averaging about 20 per cent slower than that of oxidation. On the basis of these estimates the flow of electrons over the DHA system rather than the MDHA system, which Hackett²¹ has shown to be present in potato extracts, may account for most of the transport over the AA system.

Conclusions

The results described in this paper have indicated that the concentration of AA in plant tissues is normally stabilized as a result of an equilibrium between the activity of enzymic systems oxidizing and reducing the vitamin; these mechanisms have been demonstrated in extracts from pea cotyledons and pea embryos.

A quantitative evaluation of the results obtained from the intact tissues supports the hypothesis that in some tissues (for example, potatoes) DHA may play a major role as an electron acceptor, whereas in others (peas, pea embryos) its function as an electron acceptor is relatively minor. Evidence, admittedly indirect, has been obtained indicating that in two tissues MDHA also acts as an electron acceptor, thus contributing to the electron transfer throughout the AA systems. With pea embryos as much as 30 per cent of the total respiration may be mediated via the two oxidized forms of the vitamin, although with green peas this figure is reduced by nearly one half. In this study of the pea embryo, I have not yet made a detailed analysis of the individual tissues; these results are thus a mean of the changes occurring in all the tissues (root, hypocotyl, plumule, cotyledons). It is possible that the participation of the AA system in respiration may be greater in some tissues than in others.^{19,20}

It is of interest to speculate whether the difference between the greater participation of DHA in the respiratory process in potato compared with that in peas and pea embryos is correlated with the fact that the tuber is essentially

resting tissue with a low respiration, whereas in the pea active metabolism involving either cell enlargement or cell division is in progress with a high rate of respiration. The greater participation of the AA system in the oxygen consumption of extracts compared with its role in the respiration of the intact tissue appears to be related to the greater activity of the oxidizing enzymes in the extracts: the resulting higher concentrations of DHA (and possibly MDHA) lead in turn to a greater rate of electron transport over these compounds. Thus the extent of the traffic over the AA system *in vivo* may depend primarily on the rate of oxidation of the vitamin *in vivo*. We have little direct knowledge as to whether this rate of oxidation changes markedly with different metabolic conditions or with different stages of development of the plant, although there is evidence to suggest that changes in the terminal oxidases do occur either during development¹⁹ or as a result of infection by parasites or fungi.²¹

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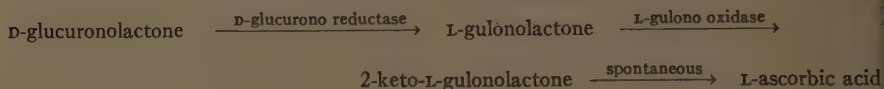
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ASPECTS OF ASCORBIC ACID BIOSYNTHESIS IN ANIMALS*

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Most species of animals can synthesize L-ascorbic acid. They synthesize the vitamin apparently from D-glucose via the D-glucuronic acid pathway of metabolism.¹⁻³ Evidence presented from this laboratory⁴⁻¹² indicates the following route of the biosynthesis of L-ascorbic acid in the animal system:



The enzyme system concerned was present in the microsomes.⁶ The mitochondrial fraction also appeared to possess some activity; the activity diminished with washing but was recovered in the washings. The activity of the thrice-washed mitochondria was about one twelfth of that of the microsomes; electron microscopic studies revealed that the washed mitochondrial fraction still contained some microsomes. Thus the observed activity of the mitochondria was very probably due to contamination by microsomes.

It has been observed in amphibians and reptiles that the enzyme system is located in the kidney microsomes and not in the liver microsomes. However, in the different avian species examined (the species that are supposed to belong to the older natural orders in the evolutionary scale, namely, the chick and the pigeon), the kidney and not the liver microsomes synthesized ascorbic acid. In the majority of the avian species studied that belong to the more recent natural orders (the Passeriformes), however, the liver tissue and not the kidney tissue synthesized ascorbic acid. In two species (the house crow and the common myna) belonging to this natural order, both the liver and the kidney tissues could effect this synthesis, the liver being more active than the kidney. However, in one species (the red-vented bulbul) belonging to this natural order, neither the liver nor the kidney could bring about the synthesis.¹³ It was indeed possible to produce scurvy in this bird species by putting it on a scorbutic diet and to cure it by administering ascorbic acid.¹⁴ In the mammalian species that can synthesize L-ascorbic acid, the enzyme system concerned is present in the liver microsomes and not in the kidney microsomes. Again, neither the liver nor the kidney microsomes of man, monkey, the Indian fruit bat, or the guinea pig could synthesize this vitamin. These results indicate that in the evolutionary ascent the enzyme originally residing in the kidney gradually passes into the liver and finally disappears from the liver also.

The microsomal enzymes, D-glucurono reductase and L-gulono oxidase, acted only on D-glucuronolactone and L-gulonolactone and not on the corresponding free acids.⁶ This was not due to the failure of penetration of the free acid through the microsomal lipid barrier since methyl glucuronate was not affected. Also a soluble enzyme system obtained from the microsomes still

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alyzed the conversion of the lactone, but not the free acid, into ascorbic acid.

Irrespective of the species examined (mouse, rat, rabbit, dog, goat, pigeon, chick, man, monkey, guinea pig, bat, and bulbul), the soluble supernate greatly inhibited the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid. The inhibitory factor has been traced to a lactonase that hydrolyzed a major part of the lactone into the free acid, the latter not being acted on by the microsomal enzyme. Sodium-L-gulonate, however, was converted to L-ascorbic acid, although to a small extent, when the lactonase of the supernate was added along with the microsomes. This was due to the reversible action of the lactonase, lactonizing a small portion of L-gulonic acid into L-gulonolactone that was then converted into L-ascorbic acid by the microsomal enzyme, the equilibrium being more in favor of the formation of the free acid than of the lactone. The microsomal formation of ascorbic acid from gulonate in the presence of the supernate can almost quantitatively be accounted for by the formation of gulonolactone from gulonate by the lactonase of the supernate.

Sodium-D-glucuronate was also converted to a small extent into L-ascorbic acid when the soluble supernate and reduced triphosphopyridine nucleotide (TPNH) were added along with the microsomes.¹² In addition to the lactonase, the soluble supernate contained a TPN-linked D-glucuronic dehydrogenase, which reduced D-glucuronic acid into L-gulonic acid; L-gulonic acid was then lactonized into L-gulonolactone by the lactonase of the supernate with its subsequent conversion into L-ascorbic acid by the action of the microsomal enzyme. It has been found in our laboratory that the supernatant lactonase that hydrolyzed D-glucuronolactone to D-glucuronic acid also lactonized, though only to a small extent, D-glucuronic acid to D-glucuronolactone. Moreover, a purified TPN-specific aldehyde dehydrogenase, free from the lactonase, has been obtained by Mano *et al.*¹⁵ from the soluble supernate that reduced D-glucuronolactone to L-gulonolactone. This also indicated the possibility of direct reduction of D-glucuronolactone (without its being hydrolyzed first to D-glucuronic acid) to L-gulonolactone in the soluble supernate.

The conversion of D-glucuronolactone into L-ascorbic acid by microsomes was strikingly stimulated by a high concentration (0.05 *M*) of sodium or potassium cyanide. Cyanide cannot be replaced by other metabolic inhibitors such as sodium azide, 2,4-dinitrophenol, phlorhizin, sodium fluoride, sodium arsenite, carbon monoxide, nor by other reducing agents such as reduced glutathione, cysteine, sodium dithionite, or sodium hypophosphite. The conversion of L-gulonolactone into L-ascorbic acid did not require the presence of cyanide. The function of cyanide in this reaction, therefore, lies apparently in the reduction of D-glucuronolactone to L-gulonolactone by D-glucuronolactonase. The mechanism of action of cyanide is very probably the release of some essential thiol groups involved in this reduction, since the enzyme concerned was inhibited by *p*-chloromercuribenzoate, which could be reversed by reduced glutathione.⁷

The intermediate product of oxidation of L-gulonolactone has been identified as 2-keto-L-gulonolactone because of the formation of the quinoxaline

derivative of the corresponding free acid by condensation with *o*-phenylenediamine and its comparison with the same derivative of an authentic sample of 2-keto-L-gulonic acid.⁹ The oxidation of L-gulonolactone into 2-keto-L-gulonolactone was also catalyzed by a thiol enzyme, namely, L-gulono oxidase. It has been observed that the stimulating effect of cyanide on the reduction of D-glucuronolactone to L-gulonolactone can be duplicated by a coupled oxidation of L-gulonolactone to 2-keto-L-gulonolactone. The oxidation of L-gulonolactone and the reduction of D-glucuronolactone might thus appear to be a coupled reaction, but this does not seem to be strictly true since this system does not produce ascorbic acid in the complete absence of oxygen. It would appear that the cofactor that was reduced in the oxidation of L-gulonolactone functioned as cyanide does in the activation of the thiol groups of D-glucurono reductase.¹² Attempts at separate identifications of D-glucurono reductase and L-gulono oxidase in the different microsomal fractions by differential centrifugation were unsuccessful; neither could these two enzymes be separated by paper electrophoresis of the soluble enzyme preparation, since both were located in the same spot.

The microsomal enzyme could not catalyze the synthesis of L-xylulose. The conversion of L-gulonate into L-xylulose was catalyzed by the diphosphopyridine nucleotide-linked (DPN-linked) L-gulonic dehydrogenase in conjunction with a decarboxylase present only in the soluble supernate.¹¹ Because the rate of formation of L-ascorbic acid from L-gulonate depended on the amount of L-gulonolactone formed by the action of the lactonase, which was also present in the soluble supernate, the synthesis of L-ascorbic acid from L-gulonate was competitive with the formation of L-xylulose at the stage of oxidation of L-gulonate to 3-keto-L-gulonate* by the DPN-linked L-gulonic dehydrogenase. The sequences of reactions involved may be represented by the scheme in FIGURE 1.

The microsomal enzyme also converted L-galactonolactone into L-ascorbic acid. The immediate product of reaction has been found to give rise to a quinoxaline derivative with *o*-phenylenediamine. This derivative had a characteristic blue fluorescence indicating that here also, as observed with L-gulonolactone, 2-keto-L-galactonolactone was very probably the immediate product of oxidation of L-galactonolactone into L-ascorbic acid by the microsomal enzyme. Sodium-L-galactonate was not converted into L-ascorbic acid by the microsomes. When the lactonase of the soluble supernate was added along with the microsomes, however, a small amount of L-ascorbic acid was produced. In this case also, as observed with sodium L-gulonate, the amount of L-ascorbic acid synthesized depended on the amount of L-galactonolactone formed from sodium-L-galactonate by the action of the lactonase, the action being reversible and the equilibrium being more toward the formation of the free acid. The pathway of formation of L-ascorbic acid from L-galactonic acid is shown in the scheme shown in FIGURE 2.

Recent work with several species (mouse, rat, rabbit, goat, pigeon, and chick) has indicated that, with the exception of the goat, the synthesis of L-ascorbic acid from L-gulonolactone by the microsomal enzyme, L-gulono

* Identified by Ashwell *et al.*¹⁰

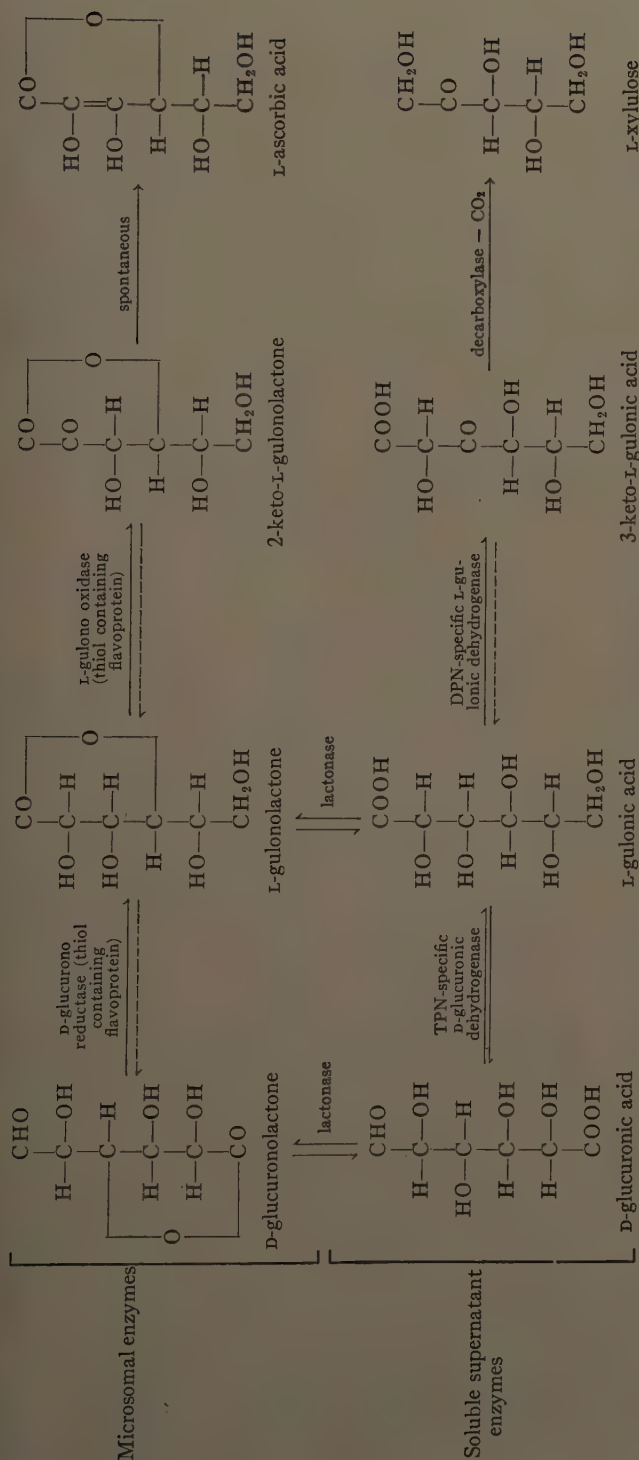


FIGURE 1. Schematic representation of the pathways of biosynthesis of L-ascorbic acid and L-xylose in the animal system.

oxidase, was stimulated by such diverse compounds as vitamin K₁, α -tocopherol, sodium pyrophosphate, α, α' -dipyridyl, 8-hydroxyquinoline, and lithium, sodium, potassium, and cesium chlorides, ribose-5-phosphate, and *o*-, *m*-, and *p*-nitrophenols. A similarly stimulating effect was obtained by the addition of the boiled supernate from the livers of the mammals and the kidneys of the birds examined. However, when the microsomes were solubilized with sodium deoxycholate, the synthesis by the soluble enzyme was no longer stimulated by any of the aforesaid agents. This lack of stimulation indicated that there was a naturally occurring substance present in the intact microsomes of all the species studied (except the goat) that probably masked the

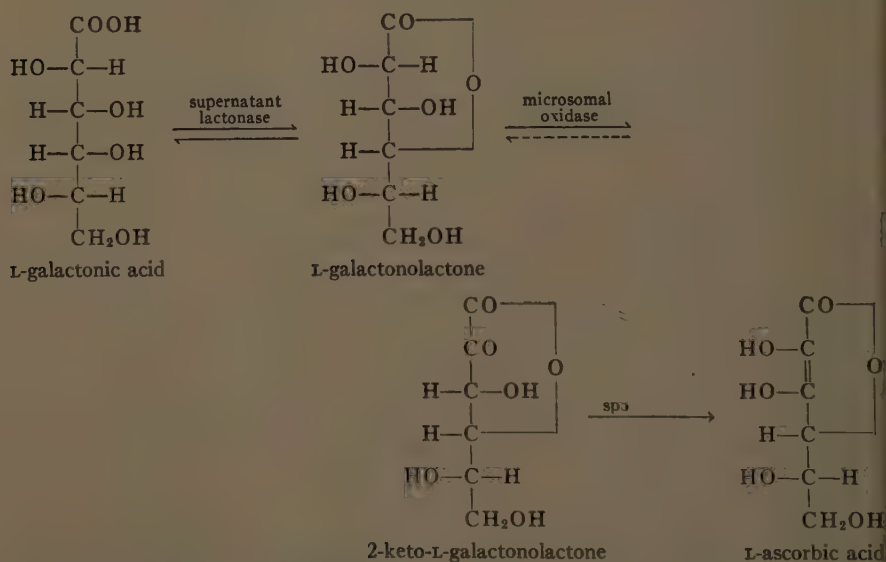


FIGURE 2. Schematic representation of the pathway of conversion of L-galactonic acid into L-ascorbic acid in the animal system.

action of L-gulonolactonase; this substance was probably counteracted by the stimulating agents, and then removed in the process of solubilization.

Dinitrophenol acted in a unique manner. It stimulated the action of the microsomal enzyme, L-gulonolactonase, in all the animals studied, including the goat. It also stimulated the solubilized enzyme. Its action seemed, therefore, to have been directly on the enzyme. Phosphorylation reaction was not involved in the conversion of L-gulonolactone into L-ascorbic acid,⁷ hence the effect of DNP as an uncoupling agent was excluded in this case.

It has been observed that the species that are unable to synthesize L-ascorbic acid (man, monkey, Indian fruit bat, guinea pig, and red-vented bulbul) contained the following enzymes in the supernate: (1) D-glucuronic dehydrogenase, reducing D-glucuronic acid into L-gulonolactone; (2) the lactonase, reversibly converting L-gulonolactone into L-gulonolactone as well as D-glucurono- lactone into D-glucuronic acid; and (3) L-gulonolactonase and the

decarboxylase converting L-gulonic acid into L-xylulose. However, the microsomal enzyme, L-gulono oxidase, which oxidizes L-gulonolactone into 2-keto-L-gulonolactone, was absent in all the aforesaid species. In view of the possibility that L-gulono oxidase might be present but not functioning normally because of the presence of some naturally occurring inhibitory substances, we incubated L-gulonolactone with the liver microsomes of man, monkey, Indian fruit bat, guinea pig, and red-vented bulbul as well as the kidney microsomes of the bat and the bulbul with the aforesaid stimulating agents; however, neither oxygen consumption nor synthesis of L-ascorbic acid was noticed. Thus the genetic defect of man, monkey, Indian fruit bat, guinea pig, and red-vented bulbul is apparently due solely to the absence of the L-gulono oxidase system, which distinguishes these species from others that can synthesize L-ascorbic acid.

This paper further describes some of the above-mentioned aspects of the mechanism of the synthesis of L-ascorbic acid in the animal system.

Methods and Materials

The separation of mitochondria, microsomes, and soluble supernate, the preparation of the soluble enzyme, the method of solvent extraction of the enzyme, the identification and the estimation of ascorbic acid, lactone, xylulose, and the various other methods and materials mentioned in this paper followed procedures previously described by Chatterjee *et al.*^{7,11,12,17}

Vitamin E deficiency was produced by placing the rats on a vitamin E-deficient diet for a period of 8 weeks.¹⁸ Vitamin A deficiency was produced according to the method of Chatterjee.¹⁹

Results and Discussion

Effect of some factors on the microsomal synthesis of L-ascorbic acid. It has previously been reported¹¹ that some chelating agents and boiled supernate stimulated the conversion of L-gulonolactone into L-ascorbic acid by rat liver and chick kidney microsomes. It has subsequently been observed that a number of other widely different substances such as 2,4-dinitrophenol (DNP), *p*-, *m*-, and *p*-nitrophenol, α -tocopherol, vitamin K₁ (Synkavite), and lithium, sodium, potassium, and cesium chlorides, sodium sulfate, ammonium chloride, ethylenediaminetetracetate (EDTA) ribose-1-phosphate, ribose-5-phosphate, xylulose-5-phosphate, and dihydroxyacetone phosphate also stimulated the synthesis. The stimulation was observed in experiments with microsomes from rat liver and chick kidney as well as from mouse liver, rabbit liver, and guinea pig kidney. The results are given in TABLE 1.

The effect of different concentrations of sodium chloride on the conversion of L-gulonolactone into L-ascorbic acid by the rat liver microsomes is shown in TABLE 2. The synthesis of L-ascorbic acid (TABLE 2) increased with an increasing concentration of sodium chloride and reached a maximum with 0.5% NaCl; above that no further stimulation was noticed.

The effect of variation of the concentration of ribose-5-phosphate on the conversion of L-gulonolactone into L-ascorbic acid by the rat liver microsomes is shown in TABLE 3. Maximum synthesis was obtained with $1 \times 10^{-3} M$ ribose-5-phosphate.

TABLE 1

STIMULATING EFFECT OF CERTAIN FACTORS ON THE CONVERSION OF L-GULONOLACTONE INTO L-ASCORBIC ACID BY THE INTACT MICROSOMES*

Factors added	Concentration used	Ascorbic acid (μ mole/mg. protein)					
		Mouse liver	Rat liver	Rabbit liver	Goat liver	Pigeon kidney	Chick kidney
None	—	Trace	0.05	0.21	0.20	0.25	0.16
2,4-Dinitrophenol	0.001 <i>M</i>	0.28	0.15	0.35	0.25	0.65	0.45
<i>o</i> -, <i>m</i> -, and <i>p</i> -Nitrophenol	0.001 <i>M</i>	0.18	0.09	0.24	0.20	0.35	0.20
Sodium pyrophosphate	0.005 <i>M</i>	0.23	0.11	0.29	0.21	0.55	0.38
α , α' -Dipyridyl	0.005 <i>M</i>	0.21	0.10	0.27	0.20	0.50	0.32
8-Hydroxyquinoline	0.001 <i>M</i>	0.22	0.12	0.30	0.20	0.53	0.35
EDTA	0.001 <i>M</i>	0.20	0.10	0.28	0.18	0.40	0.30
Reduced glutathione	0.01 <i>M</i>	0.18	0.09	0.26	0.21	0.45	0.25
Vitamin K ₁ (Synkavite)	0.5 mg.	0.25	0.10	0.20	0.19	0.24	0.18
α -Tocopherol	0.5 mg.	0.20	0.08	0.20	0.18	0.24	0.17
Coenzyme Q ₁₀ , Q ₇	0.5 mg.	0.06	0.04	0.20	0.18	0.20	0.15
LiCl, NaCl, KCl, CsCl	0.5 <i>M</i>	0.06	0.08	0.24	0.20	0.37	0.26
Ammonium chloride	0.1 <i>M</i>	0.08	0.07	0.23	0.20	0.35	0.24
Sodium sulfate	0.5 <i>M</i>	0.04	0.06	0.21	0.20	0.46	0.31
CaCl ₂ , MnCl ₂ , MnSO ₄ , CoCl ₂	0.5 <i>M</i>	Trace	0.03	0.20	0.18	0.24	0.16
Boiled supernate	100 mg. equivalent of fresh tissue	0.25	0.11	0.26	0.20	0.49	0.31
Ribose	0.001 <i>M</i>	Trace	0.05	0.21	0.20	0.25	0.16
Ribose-5-phosphate	0.001 <i>M</i>	0.24	0.10	0.27	0.20	0.50	0.30
Ribose-1-phosphate	0.001 <i>M</i>	0.12	0.07	0.22	0.20	0.30	0.22
D-Xylulose	0.001 <i>M</i>	Trace	0.05	0.21	0.20	0.25	0.16
D-Xylulose-5-phosphate†	0.001 <i>M</i>	0.23	0.10	0.27	0.20	0.50	0.30
Dihydroxy acetone phosphate	0.001 <i>M</i>	0.24	0.11	0.26	0.20	0.51	0.31
Glucose-1-phosphate, glucose 6-phosphate, fructose-1,6-diphosphate	0.001 <i>M</i>	Trace	0.05	0.21	0.20	0.25	0.16

* Test system: 0.02 *M* sodium phosphate buffer, pH 7.2, 0.005 *M* L-gulonolactone, 0.25 ml. microsomal dispersion; total volume 2.5 ml; incubated in air at 37° C. for 1 hour.

† D-Xylulose-5-phosphate was prepared *in situ* by incubating D-xylulose (0.001 *M*), soluble supernate (0.1 ml.), ATP (0.001 *M*), and MgCl₂ (0.005 *M*).

TABLE 2

EFFECT OF VARIATION OF THE CONCENTRATION OF SODIUM CHLORIDE ON THE CONVERSION OF L-GULONOLACTONE INTO L-ASCORBIC ACID BY RAT LIVER MICROSOMES*

Concentration of NaCl (<i>M</i>)	Ascorbic acid (μ mole/mg. protein)
None	0.10
0.01	0.10
0.05	0.12
0.10	0.13
0.25	0.14
0.50	0.18
1.00	0.15
2.00	0.13

* Conditions are the same as in TABLE 1.

It is of interest to report that the biosynthetic capacity of the liver microsomes from rats of the same colony was not always similar and that it varied widely from rat to rat. In some rats the synthetic capacity was found to be almost nil; in others it was as high as $0.1 \mu\text{mole/mg. protein}$. When any of the stimulating agents mentioned in TABLE 1 (for example, sodium pyrophosphate) was added to the system, however, almost a constant degree of synthesis was always obtained (TABLE 4). Liver microsomes from the mouse also behaved similarly (TABLE 4). The results indicated that the postulated in-

TABLE 3

EFFECT OF VARIATION OF THE CONCENTRATION OF RIBOSE-5-PHOSPHATE ON THE
CONVERSION OF L-GULONOLACTONE INTO L-ASCORBIC ACID BY RAT
LIVER MICROSOMES*

Concentration of ribose-5-phosphate (<i>M</i>)	Ascorbic acid ($\mu\text{mole/mg. protein}$)
None	0.03
2×10^{-4}	0.06
5×10^{-4}	0.10
1×10^{-3}	0.15
2×10^{-3}	0.14

* Conditions are the same as in TABLE 1.

TABLE 4

STIMULATING EFFECT OF SODIUM PYROPHOSPHATE ON THE CONVERSION OF
L-GULONOLACTONE INTO L-ASCORBIC ACID*

Experiment No.	No. of rats used	Ascorbic acid ($\mu\text{mole/mg. protein}$)		No. of mice used	Ascorbic acid ($\mu\text{mole/mg. protein}$)	
		Without pyrophos- phate	With pyrophos- phate		Without pyrophos- phate	With pyrophos- phate
1	6	Nil	0.13	6	Nil	0.20
2	4	0.10	0.15	6	Trace	0.21
3	6	0.05	0.14	12	0.02	0.23
4	12	0.07	0.14		—	—

* Conditions are the same as in TABLE 1.

inhibitory substance of the microsomes varied quantitatively, sometimes occurring in a very large concentration, which resulted in very great deceleration of the synthesis. When a stimulating agent was added, which probably acted by antagonizing the inhibitory substance, the inhibition of the synthesis no longer existed, and an almost constant degree of synthesis was always obtained.

The effect of added factors on the microsomal conversion of D-glucuronolactone into L-ascorbic acid. The aforesaid factors also stimulated the microsomal conversion of D-glucuronolactone into L-ascorbic acid in the presence of $0.005 M$ KCN.¹² This was most probably due to the fact that these factors stimulated the oxidation of L-gulonolactone into L-ascorbic acid, which represents the second stage of the over-all conversion of D-glucuronolactone into L-ascor-

bic acid, the first stage being reduction of D-glucuronolactone to L-gulonolactone.

The stimulating effect of the above-listed factors was found to be independent of several variations in microsomal preparation:

(1) Microsomes could be isolated from a homogenate prepared in either isotonic or hypertonic sucrose solution.

(2) Microsomes could be isolated from rats that were either well-fed or starved for 48 hours.

(3) Microsomes could be either unwashed, or washed up to 3 times with isotonic sucrose solution, or washed twice with a mixture of 0.5 *M* NaCl + 0.01 *M* sodium phosphate buffer, pH 7.4.

TABLE 5

EFFECT OF BOILED SUPERNATES FROM DIFFERENT SPECIES ON THE CONVERSION OF L-GULONOLACTONE INTO L-ASCORBIC ACID BY RAT LIVER MICROSOMES*

Source of boiled supernate	Ascorbic acid (μ mole/mg. protein)
None	0.12
Mouse liver	0.21
Rat liver	0.23
Rabbit liver	0.15
Dog liver	0.16
Goat liver	0.16
Human liver	0.23
Monkey liver	0.23
Guinea pig liver	0.18
Chick kidney	0.16
Pigeon kidney	0.18
Red-bulbul kidney	0.16
Red-bulbul liver	0.20
Indian fruit bat liver	0.23

* Conditions are the same as in TABLE 1.

(4) Microsomes could be either isolated freshly or aged by subjection to freezing at -19° C. and subsequent thawing at room temperature daily for 7 days.

The stimulating effect of the boiled supernate on the synthesis of L-ascorbic acid. As reported earlier,¹¹ the boiled supernate from all the species examined (namely, mouse, rat, rabbit, dog, goat, man, monkey, guinea pig, chick, pigeon, bulbul, and bat) have been found to be active in regard to the synthesis of ascorbic acid (TABLE 5).

Concentration of the factor present in the boiled supernate of the rat liver. Rat liver soluble supernate (20 ml., equivalent to 4 gm. liver) was kept in a boiling water bath for 5 min. with constant stirring, cooled, and centrifuged. The residue was discarded, and the supernate was dialyzed at 0° C. for 6 hours with constant stirring with three changes of distilled water (Fraction I, pale yellow).

To this dialyzed boiled supernate, sodium chloride was added to a final concentration of 5×10^{-1} *M*; this was then placed in a boiling water bath for 2 min. with constant stirring; it was then cooled and centrifuged. The residue

was discarded, and the supernate dialyzed with constant stirring at 0° C. for 4 hours against three changes of distilled water (Fraction II, faint yellow).

To 18 ml. of Fraction II were added 3 ml. of 0.2 *M* phosphate buffer (*pH* 7.8), 3 ml. of 5 per cent sodium deoxycholate solution, 28 ml. of absolute ethanol, followed by 1.2 ml. of 0.4 *M* barium acetate solution. This was kept at 37° C. for 30 min. with occasional stirring and then centrifuged. The precipitated barium salt was washed three times with distilled water, suspended in 10 ml. of distilled water, and decomposed with 0.66 ml. of 0.1 *M* sodium sulfate solution. This was allowed to stand for 15 min. and then centrifuged.

TABLE 6
CONCENTRATION OF THE BOILED SUPERNATE FROM RAT LIVER*

Fraction	Dry weight (mg.)	Ascorbic acid (amt. synthesized, μ g.)	Specific activity†
None	—	130	—
Fraction I	73.00	220	3
Fraction II	6.00	210	35
Fraction III	0.85	230	270
Fraction IV	0.60	240	400
Fraction V	0.45	240	530

* Conditions are the same as in TABLE 1.

† The specific activity is expressed in micrograms of ascorbic acid synthesized per milligram of dry weight.

TABLE 7

EFFECT OF VARIATION OF THE CONCENTRATION OF PURIFIED RAT LIVER BOILED SUPERNATE ON THE CONVERSION OF L-GULONOLACTONE INTO L-ASCORBIC ACID BY RAT LIVER MICROSOMES*

Purified boiled supernate, Fraction V (mg. dry wt.)	Ascorbic acid (μ mole/mg. protein)
None	0.10
0.15	0.12
0.30	0.13
0.45	0.14
0.60	0.14

* Conditions are the same as in TABLE 1.

The residue (BaSO_4) was discarded, and the colorless supernate was dialyzed with distilled water (Fraction III).

Fraction III was treated with 0.3 *N* NaOH at 37° C. for 18 hours, neutralized with 3 *N* HCl, and then dialyzed with distilled water to remove sodium chloride (Fraction IV).

Fraction IV was treated with *N* HCl at 100° C. for 1 hour with constant stirring, then cooled, neutralized with 3.5 *N* NaOH, and dialyzed with distilled water to remove sodium chloride (Fraction V). The results showing the increase in the specific activity at each step of the purification process are given in TABLE 6.

Effect of variation of the concentration of the purified boiled supernate. TABLE 7 shows the effect of varied concentrations of the factor (Fraction V) obtained

from rat liver boiled supernate on the synthesis of L-ascorbic acid from L-gulonolactone.

Nature of the factor present in the boiled supernate. On analysis, the concentrated boiled supernate (Fraction V) was found to contain about 50 per cent protein on the basis of dry weight. It was also found to contain phosphorus and ribose; it gave an absorption maximum at 260 m μ .

The activity of the concentrated factor (Fraction V) was not lost on treatment with pancreatic ribonuclease or with trypsin. It was lost, however, on dialysis overnight but not by dialysis for 3 to 4 hours. Yeast ribonucleic acid, purine and pyrimidine bases, and the nucleotides tested,* singly or conjointly, could not replace the factor in the boiled supernate for stimulating the conversion of L-gulonolactone into L-ascorbic acid. The activity of Fraction V could not be accounted for by its ribose-5-phosphate content, since the amount of ribose-5-phosphate (TABLE 3) required for optimum stimulation was about 20 to 25 times of that present in Fraction V. However, the possibility of the occurrence of some "active form" of ribose-5-phosphate in Fraction V, which might be the actual stimulating agent for the microsomal conversion of L-gulonolactone into L-ascorbic acid, is not excluded. This is under further investigation.

Synthesis by the solvent-extracted microsomes. Earlier it was reported¹⁷ that extraction of the microsomes with diethyl ether or petroleum ether in the presence of alcohol resulted in a decrease in the enzymic activity for the conversion of L-gulonolactone into L-ascorbic acid and that the activity could be partially restored by the addition of lipid agents such as α -tocopherol and vitamin K₁ (Synkavite) and also, although less efficiently, by the addition of a lipid extract from the microsomes. It was therefore suggested that a lipid might be a cofactor of L-gulono oxidase. However, it has since been discovered that, in addition to the lipid agents, a number of other diverse substances mentioned in TABLE 1 could partially restore the activity (TABLE 8). Thus the action of the lipid agents does not appear to be specific. It has been shown (TABLE 1) that the stimulating effect of these factors was obtained with the microsomes from the mouse, rat, rabbit, pigeon, and chick, but not from the goat (DNP being an exception).

TABLE 8, however, shows that when the goat liver microsomes were extracted with a fat solvent, the stimulating effect of the aforesaid factors became apparent. This would indicate that in the case of goat liver microsomes, probably some kind of lipid barrier protected the enzyme from a naturally occurring substance that was inhibitory to the action of the enzyme. A fat solvent removed this barrier and exposed the enzyme to the inhibitory action of the substance, thus reducing the activity of the enzyme. Since the activity of the extracted microsomes as well as the activity of the unextracted microsomes of the mouse, rat, rabbit, chick, and pigeon, (unlike that of the goat) was en-

* Tested nucleotides: ATP—adenosinetriphosphate; ADP—adenosinediphosphate; AMP—adenosinemonophosphate; GTP—guanosine triphosphate; CTP—cytidine triphosphate; CMP—cytidine monophosphate; IMP—inosine monophosphate; UTP—uridine triphosphate; UDP—uridine diphosphate; UMP—uridine monophosphate; UDPG—uridine diphosphoglucose; FAD—flavin adenine dinucleotide; FMN—flavin mononucleotide (riboflavin-5'-phosphate).

hanced by these agents, the postulated lipid barrier present in the microsomes from these species probably did not fully protect the enzyme from the action of the inhibitory substance.

TABLE 8
EFFECT OF VARIOUS FACTORS ON THE CONVERSION OF L-GULONOLACTONE INTO L-ASCORBIC ACID BY GOAT LIVER MICROSOMES*

Additions	Ascorbic acid (amt. synthesized, μ mole/mg. protein)	
	Unextracted microsomes	Extracted microsomes
None	0.18	0.06
α -Tocopherol	0.18	0.10
Vitamin K (Synkavite)	0.18	0.13
Coenzyme Q ₁₀ , Q ₇	0.18	0.08
Petroleum ether:alcohol extract of the microsomes	0.16	0.08
2,4-Dinitrophenol	0.27	0.18
Sodium pyrophosphate	0.19	0.13
Sodium chloride	0.18	0.10
EDTA	0.18	0.11
MnCl ₂	0.17	0.09
Boiled supernate	0.18	0.11
Ribose-5-phosphate	0.18	0.10

* The microsomes were extracted with a mixture of diethyl ether and alcohol (10:1) at 15° C. for 1–2 min. Other conditions are given elsewhere.¹⁷

TABLE 9
EFFECT OF VARIOUS FAT SOLVENTS ON THE ACTIVITY OF RAT LIVER MICROSOMES FOR THE CONVERSION OF L-GULONOLACTONE INTO L-ASCORBIC ACID*

Solvent used	Time of treatment (min.)	Ascorbic acid (μ mole/mg. protein)	
		Without vitamin K ₁	With vitamin K ₁
None		0.08	0.13
Diethyl ether:ethyl alcohol (10:1)	1	0.02	0.10
Diethyl ether:ethyl alcohol (10:1)	5	Trace	0.05
Diethyl ether:ethyl alcohol (10:1)	15	Nil	Nil
Acetone (20 per cent)	5	Nil	Nil
Butanol (20 per cent, v/v)	2	Trace	0.03
Chloroform (20 per cent, v/v)	1	Nil	Nil

* Conditions are the same as in TABLE 8.

The stimulation of the solvent-treated microsomes by the agents mentioned above depended on the nature of the treatment used. On subjecting the microsomes to a mild treatment with the fat solvent (for example, by shaking the microsomal dispersion in the presence of alcohol with diethyl ether or petroleum ether for 1 to 2 minutes at -18° C.), the microsomal enzyme was only partially inactivated, and the degree of stimulation approached that obtained with the untreated microsomes (TABLE 9). The degree of stimulation de-

creased as the time of shaking the microsomal dispersion with the ether-alcohol mixture was increased indicating inactivation of the microsomal enzyme during this treatment. Shaking the microsomes for a considerably longer period (for example, 10 to 15 min.), resulted in complete inactivation of the enzyme, and, in this experiment, no stimulating effect of the added agents was observed. Similar results were also obtained by treating the microsomal dispersion with acetone, chloroform, or *n*-butanol (TABLE 9). Thus it became apparent that the microsomes that had been only partially inactivated by treatment with fat solvents did respond to the stimulating effect of the agents, the degree of stimulation being similar to that observed with the corresponding untreated microsomes. The enhancement of the synthesis of L-ascorbic acid with the solvent-treated microsomes by any of the agents mentioned before (TABLE 8) could therefore be explained simply as a stimulation of the residual enzyme

TABLE 10
EFFECT OF VARIOUS FACTORS ON THE CONVERSION OF L-GULONOLACTONE INTO
L-ASCORBIC ACID BY SOLVENT-EXTRACTED MICROSOMES AND SOLUBLE
ENZYME PREPARATION*

Factors	Ascorbic acid (amt. synthesized, μ mole/mg. of protein)							
	Extracted mouse liver		Extracted rat liver		Extracted goat liver		Extracted chick kidney	
	Microsomes	Soluble enzyme	Microsomes	Soluble enzyme	Microsomes	Soluble enzyme	Microsomes	Soluble enzyme
None	Trace	0.05	0.02	0.03	0.08	0.12	0.04	0.08
Vitamin K ₁	0.12	0.05	0.09	0.03	0.14	0.12	0.08	0.08
Microsomal lipid extract	0.04	0.03	0.03	0.02	0.09	0.10	0.05	0.08
Sodium pyrophosphate	0.11	0.06	0.10	0.04	0.13	0.13	0.10	0.09
EDTA	0.12	0.06	0.10	0.04	0.13	0.12	0.10	0.09
Sodium chloride	0.08	0.05	0.05	0.03	0.10	0.12	0.06	0.08
Boiled supernatant	0.12	0.05	0.09	0.03	0.11	0.12	0.08	0.08
Ribose-5-phosphate	0.12	0.05	0.10	0.03	0.11	0.12	0.09	0.08

* Conditions are the same as in TABLE 8.

activity by these agents. It has been observed that in the absence of alcohol, the enzymic activity of the microsomes was not decreased by treatment with petroleum ether, isooctane, or cyclohexane. Thus treated, the microsomes behaved like untreated microsomes toward the aforesaid stimulatory agents.

The reactivation of the solvent-treated microsomes by the lipid agents mentioned above, namely α -tocopherol, vitamin K₁, or the microsomal lipid extract could also be explained, therefore, simply as the stimulating effect of these agents and need not be ascribed to the possible replacement of some essential lipid cofactor removed during solvent extraction. This was confirmed by the observation that extraction of the enzyme solution prepared from the microsomes, irrespective of species, with diethyl ether or petroleum ether in the presence of alcohol results in a decrease in the enzymic activity, but in this case the activity was not restored by any of the aforesaid agents, including the lipid factors (TABLE 10). TABLE 11 shows that, irrespective of the species, the synthesis of ascorbic acid by the soluble enzyme was not stimulated by

any of the factors mentioned above. Apparently in solubilizing and purifying the enzyme, the naturally occurring inhibitory substance present in the intact microsomes was removed. The added factors stimulated the enzyme activity of the microsomes by antagonizing the inhibitory substance; also in the experiment with solvent-extracted microsomes, the enhancement of the synthesis was a mere stimulation of the residual enzyme activity. In the soluble enzyme, the inhibitory substance was apparently absent, and hence none of the factors could stimulate the synthesis, even after solvent treatment. This irreversible loss in the activity of the soluble enzyme by treatment with fat solvents appeared to be due to denaturation as indicated by its decreased solubility.

Mechanism of stimulation of the microsomal synthesis of L-ascorbic acid by the added factors. The factors mentioned in TABLE 1 enhanced the oxidation of L-gulonolactone by the intact microsomes, leading to the formation of L-ascorbic acid. These agents, however, did not stimulate the oxidation by the deoxy-

TABLE 11

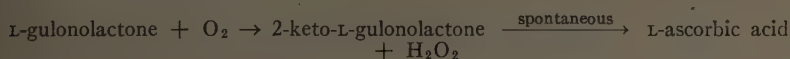
EFFECT OF VARIOUS FACTORS ON THE CONVERSION OF L-GULONOLACTONE INTO L-ASCORBIC ACID BY RAT LIVER MICROSOMES AND SOLUBLE ENZYME*

Factors	Ascorbic acid (amt. synthesized, μ mole/mg. protein)	
	Microsomes	Soluble enzyme
None	0.08	0.13
Vitamin K ₁	0.12	0.13
2,4-Dinitrophenol	0.18	0.20
Sodium pyrophosphate	0.13	0.14
Sodium chloride	0.11	0.13
Boiled supernate	0.12	0.13
Ribose-5-phosphate	0.12	0.13

* Conditions are the same as in TABLE 1.

cholate-soluble microsomes. As stated before, this indicated that in the intact microsomes the enzyme was probably masked by some inhibitory substance and that the added factors acted indirectly by antagonizing the inhibitory substance, thus leading to a net stimulation in the synthesis, the inhibitory substance being removed in the process of solubilizing the enzyme from the microsomes. Had the agents any stimulating effect on the enzyme per se, they would have stimulated also the synthesis by the soluble enzyme.

The possibility that the stimulating effect of the aforesaid factors might be due to the protection of the synthesized ascorbic acid from oxidative breakdown does not exist in view of the fact that washed microsomes (washed twice with a mixture of isotonic sucrose and 0.5 M NaCl) did not oxidize added L-ascorbic acid, and the stimulating effect was equally observed with the washed microsomes. In the presence of the stimulating agents, oxygen consumption was actually increased with proportional increase in the ascorbic acid formation, according to the reaction:



The agents that stimulated the microsomal synthesis of L-ascorbic acid were widely divergent in nature; perhaps the different agents acted in different ways, all leading to a net increase in the synthesis. α -Tocopherol, vitamin K₁, and other lipid agents might have acted as antioxidants, inhibiting the formation of lipid-peroxides²⁰ in the microsomes, which otherwise might normally inhibit the synthesis by oxidizing the essential thiol groups involved in the enzyme.

It might be that the chelating agents and reduced glutathione acted by releasing the essential thiol groups masked by some naturally occurring inhibitory substance. In order to investigate whether the inhibitory substance could be extracted from the microsomes by pretreatment with the chelating agents, rat liver microsomes were incubated with EDTA (0.01 *M*), centrifuged, and the sedimented microsomes washed free of EDTA by dialysis, then incubated as usual with L-gulonolactone. It was observed that the biosynthetic capacity of the EDTA-treated microsomes was increased by about 25 per cent, but the activity was not further increased by the addition of EDTA. This would indicate possible removal of the inhibitory substance by pretreatment with EDTA. The EDTA extract of the microsomes (dialyzed free of EDTA) did not inhibit the synthesis, however, either by the EDTA-treated microsomes or by the untreated microsomes. It is possible, however, that EDTA antagonized the action of the inhibitory substance by irreversible chelation. The synthesis by the microsomes, which had been pretreated with sodium pyrophosphate, was stimulated further by the addition of pyrophosphate; this was also true with 0.5 *M* NaCl-pretreated microsomes.

The inhibitory substance was apparently removed by treating the microsomes with sodium deoxycholate. However, again adding back the deoxycholate-insoluble portion of the microsomes (which consisted chiefly of ribonucleoprotein particles) to the deoxycholate-soluble portion did not result in any inhibition of the synthesis. It is possible that the L-gulono oxidase in the intact microsomes was masked by some ribonucleoprotein, which once released could not be reconstituted under the experimental conditions used.

However, the nature of the inhibitory substance present in the microsomes as well as the mechanism of action of the stimulating agents was not clear, and the investigation continues. The mechanism of action of 2,4-dinitrophenol (DNP) appeared to differ from that of the other agents used since DNP stimulated the synthesis by microsomes as well as by soluble enzyme; its action therefore was more direct.

Synthesis of L-ascorbic acid by microsomes from vitamin-deficient rats. We have previously reported¹⁷ that the enzymic activity of the liver microsomes for the conversion of L-gulonolactone into L-ascorbic acid was significantly decreased in vitamin E deficiency and that the activity could be restored by addition of α -tocopherol or vitamin K₁. Similar reactivation by α -tocopherol has also been obtained by Carpenter *et al.*,²⁰ who observed an increased lipid-peroxide formation in the microsomes from vitamin E-deficient rats; correlating this with the decreased formation of ascorbic acid in vitamin E deficiency, they concluded that the agents used, including α -tocopherol, acted by inhibiting the peroxide formation. Results obtained in our laboratory, however, showed that compounds other than antioxidants also equally stimulated the synthesis

of L-ascorbic acid by the microsomes from vitamin E-deficient rats (TABLE 12). Thus the action of the antioxidants did not appear to be specific. Similar results have also been obtained with the microsomes from vitamin A-deficient, thiamine-deficient, and riboflavin-deficient rats (TABLE 12). In each test, the activity of the microsomes could be restored by feeding the respective vitamins to the deficient rats. In the vitamin E-deficiency experiments, α -tocopherol could be substituted by methylene blue *in vivo* for restoration of the enzymic activity, but unlike α -tocopherol, methylene blue could not restore the microsomal activity when added *in vitro*.

Solubilization and concentration of the microsomal enzyme catalyzing the synthesis of L-ascorbic acid. It has been reported¹² that the microsomes could be effectively solubilized with sodium deoxycholate and that the enzyme could be further concentrated by fractionation with ammonium sulfate. The fraction

TABLE 12

CONVERSION OF L-GULONOLACTONE INTO L-ASCORBIC ACID BY MICROSOMES FROM VITAMIN-DEFICIENT RATS*

Factors added	Ascorbic acid (amt. synthesized, μ mole/mg. protein)							
	Vitamin E		Vitamin A		Thiamine		Riboflavin	
	Deficient	Pair-fed control	Deficient	Pair-fed control	Deficient	Pair-fed control	Deficient	Pair-fed control
None	0.02	0.08	Trace	0.03	0.06	0.10	0.02	0.11
Tocopherol	0.08	0.12	0.03	0.06	0.09	0.12	0.06	0.12
Vitamin K ₁	0.10	0.13	0.04	0.08	0.10	0.14	0.08	0.13
Sodium pyrophosphate	0.10	0.14	0.04	0.08	0.10	0.14	0.10	0.14
Sodium chloride	0.08	0.12	0.03	0.06	0.09	0.12	0.06	0.12
NaCl ₂	0.06	0.10	0.02	0.05	0.08	0.11	0.05	0.11
Boiled supernate	0.10	0.14	0.04	0.08	0.10	0.14	0.10	0.14

* Conditions are the same as in TABLE 1.

precipitating out at 15 to 20 per cent saturation with ammonium sulfate contained the highest activity in terms of micrograms of ascorbic acid synthesized per milligram of protein. Solubilization of the microsomes and fractionation with ammonium sulfate in the presence of a reducing substance such as potassium cyanide or reduced glutathione did not give better results.

Attempts to concentrate the enzyme by extracting the lyophilized microsomes with dry *n*-butanol at -15° C. were not successful; the activity of the microsomes, however, was not lost in this treatment. Replacement of *n*-butanol by acetone in this process resulted in the inactivation of the enzyme. Treatment with ethanol at -10° C. in the presence of 0.5 *M* NaCl also resulted in loss of activity. Efforts to concentrate further the ammonium sulfate-fractionated soluble enzyme preparation by column chromatography on silica, alumina, α -cellulose, carboxymethyl cellulose, or DEAE-cellulose* or on C-50 and IRC-120 resins were not successful. The enzyme was not adsorbed by cellulose, but it was irreversibly adsorbed by silica gel or DEAE-

* Diethylaminoethyl cellulose.

cellulose. The enzyme could, however, be separated by electrophoresis on paper (Whatman No. 3), but in this case also the enzyme was largely adsorbed by the paper and could not be efficiently eluted. It should be mentioned that the particular band on the paper that catalyzed the conversion of L-gulonolactone into L-ascorbic acid also catalyzed the conversion of D-glucuronolactone into L-ascorbic acid. Thus D-glucurono reductase and L-gulono oxidase could not be separated by paper electrophoresis under the conditions used.

Missing step in man, monkey, Indian fruit bat, guinea pig, and red-vented bulbul. Man, monkey, Indian fruit bat, guinea pig, and red-vented bulbul cannot synthesize L-ascorbic acid and are dependent on an exogenous supply of the vitamin. Investigations were therefore carried out to determine the exact biochemical step missing in these species for the synthesis of L-ascorbic acid with special reference to the pathways involved in the synthesis as represented in FIGURE 1.

TABLE 13
EFFECT OF LACTONASE FROM THE SOLUBLE SUPERNATE OF DIFFERENT SPECIES
ON THE HYDROLYSIS AND FORMATION OF D-GLUCURONOLACTONE
AND L-GULONOLACTONE*

Source of supernatant lactonase	Lactone formed from		Lactone hydrolyzed from	
	Sodium-D-glucuronate (40 μ mole)	Sodium-L-gulonate (50 μ mole)	D-Glucuronolactone (20 μ mole)	L-Gulonolactone (20 μ mole)
	μ mole	μ mole	μ mole	μ mole
Human liver	1.8	2.2	14.0	16.0
Monkey liver	2.8	1.5	15.0	13.0
Indian fruit bat liver	1.7	2.8	16.0	15.0
Indian fruit bat kidney	1.2	1.5	13.0	13.0
Guinea pig liver	1.0	1.0	12.0	12.0
Red-vented bulbul liver	1.2	1.6	13.0	13.0
Red-vented bulbul kidney	1.0	1.5	12.0	13.0

* Conditions are given elsewhere.¹¹

Enzymes in the soluble supernatant. (1) *Lactonase*: It has been observed that the soluble supernates from the livers of man, monkey, Indian fruit bat, guinea pig, and red-vented bulbul, as well as from the kidneys of the bat and the bulbul contained glucuronolactonase and gulonolactonase, reversibly converting D-glucuronolactone and L-gulonolactone to the corresponding free acids, the equilibrium being much more favorable for the formation of the free acid (TABLE 13). Yamada²¹ purified a lactonase from the soluble supernate that hydrolyzed both D-glucuronolactone and L-gulonolactone. It therefore appears that glucuronolactonase and gulonolactonase as reported in this paper may be the same enzyme. The reversibility of the enzyme could not however be demonstrated by Yamada,²¹ probably due to the fact that they did not use any trapping agent and because the equilibrium of the reaction was far more toward hydrolysis in the absence of a trapping agent, the lactone could not accumulate.

(2) *D-Glucuronic acid dehydrogenase*: It has been observed that the soluble supernates from the livers of man, monkey, Indian fruit bat, guinea pig, and

red-vented bulbul as well as from the kidneys of the bat and the bulbul contained D-glucuronic acid dehydrogenase, the enzyme reducing D-glucuronic acid to L-gulononic acid (TABLE 14). This enzyme was assayed indirectly by coupling it with the lactonase present in the supernate and with added goat liver microsomes containing L-gulonolactone oxidase. The amount of L-ascorbic acid formed in this system would represent the amount of L-gulonolactone formed from D-glucuronate by the action of D-glucuronic acid dehydrogenase. L-Gulonolactone was then converted into L-gulonolactone by the lactonase of the supernate and

TABLE 14
CONVERSION OF SODIUM GLUCURONATE INTO L-ASCORBIC ACID*

Source of D-glucuronic dehydrogenase and lactonase	Source of L-gulonolactone oxidase	L-Ascorbic acid (amt. synthesized, μ mole)
Human liver	Goat liver	0
Monkey liver	Goat liver	0.08
Indian fruit bat liver	Goat liver	0.27
Indian fruit bat kidney	Goat liver	0.38
Guinea pig liver	Goat liver	0.17
Red-vented bulbul liver	Goat liver	0.31
Red-vented bulbul kidney	Goat liver	0.12
	Goat liver	0.08

* Conditions are given elsewhere.¹²

TABLE 15
CONVERSION OF SODIUM-L-GULONATE INTO L-XYLULOSE*

Source of L-gulonolactone dehydrogenase and decarboxylase	L-Xylulose formed (μ mole)
Human liver	0.14
Monkey liver	0.46
Indian fruit bat liver	0.33
Indian fruit bat kidney	0.22
Guinea pig liver	0.40
Red-vented bulbul liver	0.26
Red-vented bulbul kidney	0.10

* Conditions are given elsewhere.¹¹

was oxidized to L-ascorbic acid by L-gulonolactone oxidase of the goat liver microsomes.

3) *L-Gulonolactone dehydrogenase and decarboxylase*: These two enzymes have also been found in species unable to synthesize L-ascorbic acid (TABLE 15). The enzyme system was assayed by estimating the amount of DPN reduced with corresponding formation of L-xylulose, using L-gulonolactone as the substrate. *Enzymes in the microsomes*. It has been found that the liver microsomes from man, monkey, Indian fruit bat, guinea pig, and red-vented bulbul as well as the kidney microsomes of the bat and the bulbul could not convert D-glulonolactone or L-gulonolactone into L-ascorbic acid. As previously mentioned,¹¹ this was probably due to the absence of L-gulonolactone oxidase. It has already been mentioned in this paper that the activity of the microsomes from species that can synthesize L-ascorbic acid (the mouse, rat, rabbit, pigeon, and

chick) was stimulated by substances such as DNP, sodium pyrophosphate, and vitamin K₁, owing apparently to their counteracting a naturally occurring inhibitory substance present in the microsomes of these species. In view of the possibility that the presence of L-gulonolactone oxidase in the microsomes from man, monkey, bat, guinea pig, and bulbul might be completely masked by the presence of some strong inhibitory substance, the microsomes or the solubilized microsomes from these species were incubated with L-gulonolactone in the presence of DNP, sodium pyrophosphate, and vitamin K₁; however, no oxygen consumption leading to the formation of L-ascorbic acid was observed. Also, addition of the microsomes or the solubilized microsomes from these species to the solubilized enzyme from rat liver microsomes did not inhibit the synthesis.

Baker *et al.*²² claimed that they obtained increased ascorbic acid excretion by feeding D-glucuronolactone to human subjects, but the results could not be reproduced in this laboratory.

Since the presence of other enzymes involved in the synthesis of L-ascorbic acid (namely, D-glucuronic acid dehydrogenase and gulonolactonase) had been ascertained, the genetic defect in man, monkey, Indian fruit bat, guinea pig, and red-vented bulbul is apparently due only to the absence of the L-gulonolactone oxidase system (more recent experiments indicate that probably the D-glucuronic acid reductase is also lacking in these species).

Synthesis of L-ascorbic acid by Chloretone-fed rats. When administered to rats, Chloretone and other narcotics strikingly enhanced the urinary excretion of L-ascorbic acid. When added *in vitro*, however, Chloretone did not stimulate the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid by either the microsomes or the homogenate. The activity of the enzymes examined *in vitro* (D-glucuronic dehydrogenase, L-gulonic dehydrogenase, the supernatant lactonase, D-glucuronic reductase, and L-gulonolactone oxidase) has been found to be unenhanced in Chloretone-fed rats.

Let us assume that the sequence of reactions in the formation of L-ascorbic acid was the same *in vivo* as indicated by *in vitro* experiments (FIGURE 1) with animals treated with narcotics and other toxic agents. Since the response to those toxic agents could lead to increased formation of D-glucuronic acid and its corresponding lactone, the enhanced synthesis of L-ascorbic acid could be attributed to the increased concentration of the appropriate substrate.

Conclusion

The synthesis of L-ascorbic acid from L-gulonolactone by microsomes of the species examined (mouse, rat, rabbit, goat, chick, and pigeon), with the exception of the goat, was stimulated by a number of diverse substances, such as α -tocopherol, vitamin K₁, some chelating agents, alkali metal salts, pentose phosphates, and by the boiled supernates obtained from the species examined. The stimulating effect of these substances was very probably due to their counteracting in various ways a naturally occurring inhibitory substance present in the intact microsomes of the species concerned. When the microsomes were solubilized with sodium deoxycholate, the synthesis by the soluble enzyme was no longer stimulated by any of the aforesaid agents, indicating that the inhibitory substance was removed in the process of solubilization.

2,4-Dinitrophenol stimulated the enzymic activity of goat liver microsomes as well as the soluble enzyme from all the species examined; its action on the enzyme was therefore probably more direct.

It was observed that those species unable to synthesize L-ascorbic acid (namely, man, monkey, Indian fruit bat, guinea pig, and red-vented bulbul) contained all the enzymes in the soluble supernate, converting D-glucuronate into L-gulonolactone as well as to L-xylulose; however, the microsomal enzyme, L-gulono oxidase, which converts L-gulonolactone into 2-keto-L-gulonolactone, was absent. This genetic defect distinguished these species from the other species that can synthesize L-ascorbic acid.

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ASPECTS OF ASCORBIC ACID BIOSYNTHESIS IN PLANTS

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Ascorbic acid is present in most, if not all, higher plants. Synthesis occurs concomitantly with germination of the seed and proceeds most rapidly in the actively growing regions. It accumulates in certain portions of the plant, notably the fruit. From the time of fruit-set to the onset of ripening, the ascorbic acid content may increase manifold. Characteristic ascorbic acid-accumulating fruits include tomato (*Solanum lycopersicum*), strawberry (*Fragaria*), rose hip (*Rosa*), walnut (*Juglans regia*), and West Indian cherry or acerola (*Malpighia punicifolia*). The natural sugars are usually considered to be the ultimate precursors of ascorbic acid, since they produce an increase in this acid when administered through the conductive tissues or the roots. Sucrose is often singled out as the principal precursor, and a quantitative relationship seems to exist between these two substances under a variety of experimental conditions. Mapson,¹ Metzner,² and Åberg³ have presented the details of these considerations in their reviews of ascorbic acid in plants.

At this laboratory, interest in the nature of the accumulation of L-ascorbic acid in ripening fruits led to the present experiments in which C¹⁴-labeled sugars and sugar derivatives were administered to detached ripening strawberries in order to establish the nature of L-ascorbic acid formation. Isherwood *et al.*⁴ had already published a nonisotopic study on the comparative aspects of ascorbic acid formation in the cress seedling and the rat. King and his colleagues had demonstrated the synthesis of L-ascorbic acid from C¹⁴-labeled sugars in the albino rat.⁵⁻⁸ Suggestions that similar C¹⁴-labeled sugar experiments be extended to plant tissues lay in the literature,^{4,9} but no attempt had been made to carry out such a study. Seegmiller *et al.*¹⁰ had already demonstrated the convenience of administering labeled sugars to detached strawberries in order to study pectin biosynthesis. By joining their procedure with techniques described by King and his colleagues, an experimental approach to the study of the formation of L-ascorbic acid in plant tissues was effected.

Aspects with C¹⁴-Labeled D-Glucose

D-Glucose-1-C¹⁴ was administered to individual, detached, ripening strawberries by the injection of a 1 per cent solution of the radioactive sugar into the berry receptacle or by uptake through the severed stem. Both methods gave similar results. After 23 to 120 hours, each berry was separated into its constituent parts, as diagrammed in FIGURE 1. The ascorbic acid was recovered in amounts ranging from 1 to 5 mg. by means of a Dowex 1 (formate) exchange resin with a 0 → 3 N formic acid gradient. Aliquots were titrated with 2,6-dichlorophenolindophenol to locate the ascorbic acid-containing fractions. Nonisotopic L-ascorbic acid was added to the combined fractions representing the ascorbic acid peak and, after evaporation of the solution to

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dryness, the diluted ascorbic acid was recrystallized several times from methanol-ether and glacial acetic acid until the specific activity no longer changed. The final recrystallized product was degraded chemically, as described by Horowitz *et al.*^{5,6} to determine the relative activity of the individual carbon atoms.

The finding fully expected was that D-glucose-1- C^{14} would be converted to L-ascorbic acid labeled principally in carbon 6, since the predicted pathway involved an oxidation of carbon 6 of D-glucose to form D-glucuronolactone.

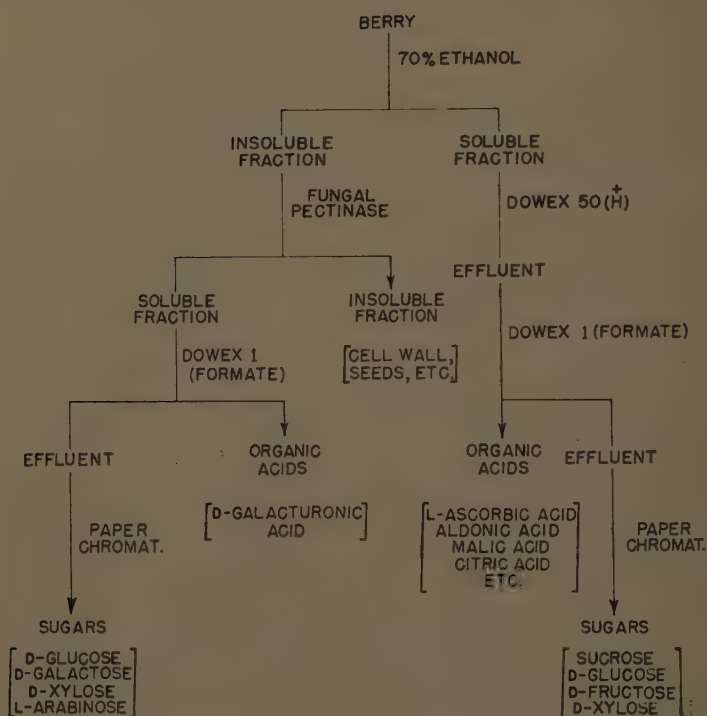


FIGURE 1. Fractionation of individual strawberries labeled with C^{14} -containing compounds.

which was then converted to L-ascorbic acid with an inversion of the carbon chain. Surprisingly enough, it was not carbon 6 but carbon 1 that retained the greatest activity (TABLE 1).¹¹ Subsequent experiments with D-glucose-2- C^{14} and D-glucose-6- C^{14} confirmed the nature of this original discovery and established the fact that the entire carbon chain of glucose was utilized in the conversion.^{11,12} The amount of C^{14} converted to L-ascorbic acid varied from 0.001 to 0.02 per cent of the administered sugar, depending upon experimental conditions. In all of the experiments a characteristic pattern of isotope redistribution between opposite halves of the six-carbon chain was noted. Thus, with D-glucose-1- C^{14} , about 15 to 19 per cent of the C^{14} in the ascorbic acid always appeared in carbon 6. The observation was not unique. Similar patterns of C^{14} distribution have been reported in other six-carbon products of

hexose metabolism in plant tissues.^{10,13-15} These patterns imply the splitting and recombination of at least 30 to 40 per cent of the hexose during its conversion.¹⁶ Presumably this results from the cleavage of hexose diphosphate to triose phosphate, followed by resynthesis of the hexose diphosphate from partially equilibrated triose fragments. This redistribution occurs very quickly after the labeled sugar has been administered,¹³ and appears to be limited to processes that affect the labeled sugar as it enters the hexose phosphate pool of the plant tissue. In this respect, ascorbic acid synthesis is very intimately associated with the hexose phosphate pool.

When D-glucose-6-C¹⁴ was simultaneously administered by injection into 3 green strawberries and they were allowed to ripen for 17, 41, and 65 hours respectively, the C¹⁴ redistribution in the ascorbic acid from the 17-hour experiment had already reached a limiting value that changed very little during

TABLE 1
DISTRIBUTION OF RADIOACTIVITY IN ASCORBIC ACID RECOVERED FROM
LABELED STRAWBERRIES

Label, initial berry color, and length of experiment (hours)	Percentage of C ¹⁴ in each carbon (sum of all 6 carbons = 100 %)					
	C-1	C-2*	C-3	C-4*	C-5	C-6
<i>Glucose-1-C¹⁴</i>						
Green 42	55	19	4	1	2	19
White 40	67	1	10	7	1	14
Pink 24	66	1	14	3	1	15
Red 24	56	9	18	2	2	15
Red 120	62	6	9	4	4	19
<i>Glucose-2-C¹⁴</i>						
White 23	0	73	0	12	10	5
White 46	0	69	6	11	12	2
<i>Glucose-6-C¹⁴</i>						
Green 47	24	1	1	2	2	73

* Values obtained by difference, C-(1+2) - C-1 and C-(4+5) - C-5.

succeeding 47 hours (TABLE 2). A slow but continuous redistribution was observed in the glucose moiety of sucrose over the same period. A comparison of the amount of C¹⁴ in sucrose with that in L-ascorbic acid and D-galactonic acid (derived from the pectin) revealed that sucrose lost activity while the other two acids gained during the period of the experiment.

Aspects with D-Galactose-1-C¹⁴

The ascorbic acid from strawberries labeled with D-galactose-1-C¹⁴ contained about the same order of activity as the ascorbic acid from strawberries administered D-glucose-1-C¹⁴. There was, however, a noticeable increase in the redistribution of C¹⁴ from carbon 1 into carbon 6 (TABLE 3). This increase was reflected by the sucrose-derived D-glucose patterns of the same berries.^{11,15} It was apparent from the sucrose data that D-galactose was converted to the glucose configuration without prior degradation of the carbon chain, probably via a mechanism involving the uridine diphosphate derivatives of these

sugars.¹⁷ Subsequent conversion of the glucose derivative to hexose phosphate and then to ascorbic acid would best fit the data. The increased redistribution of C¹⁴ in the ascorbic acid merely emphasized the nature of the path D-galactose must travel. Since glucose-1-phosphate as well as glucose-6-phosphate participated in this conversion, a greater opportunity for equilibration

TABLE 2
DISTRIBUTION OF RADIOACTIVITY IN PRODUCTS RECOVERED FROM
D-GLUCOSE-6-C¹⁴-LABELED STRAWBERRIES WITH TIME*

Product (hours)	Percentage of C ¹⁴ in each carbon (sum of all 6 carbons = 100%)					
	C-1	C-2	C-3	C-4	C-5	C-6
L-Ascorbic acid						
17	19	19	2	1	1	78
41	19	19	1	5	5	74
65	20	20	2	1	1	77
D-Glucose from sucrose						
17	14	1	1	1	0	85
41	17	2	2	2	0	79
65	16	5	5	2	0	76
D-Galacturonic acid from pectin						
17	16	2	2	2	2	81
41	19	2	2	2	2	78
65	18	2	2	2	2	80

* F. A. Loewus and S. Kelly, unpublished data.

TABLE 3
DISTRIBUTION OF RADIOACTIVITY IN PRODUCTS RECOVERED FROM
D-GALACTOSE-1-C¹⁴-LABELED STRAWBERRIES

Initial berry color and length of experiment (hours)	Percentage of C ¹⁴ in carbon 1 and carbon 6 (sum of all 6 carbons = 100%)					
	L-Ascorbic acid		D-Glucose from sucrose		D-Galacturonic acid from pectin	
	C-1	C-6	C-1	C-6	C-1	C-6
White 24	45	41	84	11	—	—
Green 64	47	39	78	14	67	21
White 40	65	23	78	14	72	23
White 47	67	25	80	15	68	25

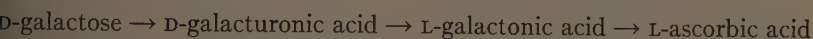
with triose phosphate was presented to the carbon chain during its utilization. The tracer studies with D-galactose suggest that this sugar is utilized for ascorbic acid synthesis only after it has been converted to a derivative of D-glucose in the strawberry.

Comparative Aspects of Ascorbic Acid Synthesis

The experiments just described made it quite apparent that the observation of C. G. King with terminally labeled D-glucose administered to rats would not be duplicated in the strawberry. One began to wonder whether the straw

erry was unique in the plant kingdom and whether it truly represented the normal path of ascorbic acid biosynthesis in higher plants. To test this, the glucose-1- C^{14} experiment was repeated on three-day-old cress seedlings (*Lepidium sativum*). Perhaps it should be noted here that plant seeds are low in ascorbic acid. Upon germination, however, they can synthesize it quite rapidly from carbohydrate reserves. When the cress seedlings were placed in a sterile solution of D-glucose-1- C^{14} , the ascorbic acid of the seedlings became radioactive. Over 70 per cent of the C^{14} was located in carbon 1, quite the same result as had been obtained in the strawberry.¹⁸

Lepidium was the plant used by Isherwood *et al.* in their comparative study of plants and animals. They noted a substantial increase in the ascorbic acid content as compared to water controls when the seedlings were placed in solutions of D-glucuronolactone, D-galacturonic acid methyl ester, L-gulonolactone and, especially, L-galactonolactone. They further demonstrated the *in vivo* reduction of D-galacturonic acid methyl ester to L-galactonolactone and the oxidation of the latter to ascorbic acid by plant extracts. These observations led them to propose the following scheme of ascorbic acid biosynthesis in plants:



This scheme demanded that the carboxyl carbon of ascorbic acid be derived from carbon 6 of galactose and that the six-carbon chain remain intact throughout the conversion. The tracer data revealed that the carboxyl carbon was derived from carbon 1 of D-glucose or D-galactose, while carbon 6 remained a primary alcohol function. Seegmiller *et al.*¹⁰ had shown that both sugars were converted to D-galacturonic acid in strawberry pectin via a pathway involving the oxidation of carbon 6. Experiments in which both ascorbic acid and galacturonic acid were recovered from the same D-galactose-1- C^{14} labeled berry (TABLE 3) revealed that both acids had their greatest amount of C^{14} in carbon 6. If D-galacturonic acid as the intermediate leading to ascorbic acid had been synthesized from D-galactose (or even D-glucose) by the same process as the one leading to pectin-derived D-galacturonic acid, one would expect D-glucose-1- C^{14} or D-galactose-1- C^{14} to label the strawberry ascorbic acid in carbon 6 rather than in carbon 1 as was observed.

Quite recently, experiments were performed* in which D-glucose-6- t (obtained from H. S. Isbell, National Bureau of Standards, Washington, D.C.) was introduced through the detached stems of individual ripening strawberries. After 40 to 60 hours each berry was separated into its constituent fractions by the methods previously used in the C^{14} experiments. Ascorbic acid was recovered as a prominent radioactive peak from a Dowex-1-formate exchange column by gradient elution with a 0 \rightarrow 0.06 *N* formic acid gradient. Nearly 90 per cent of the radioactivity found in the eluate fractions that contained ascorbic acid was retained in the L-ascorbic acid after dilution with nonisotopic material and recrystallization from glacial acetic acid. The nonexchangeable tritium retained in the ascorbic acid corresponded to 0.05 to 0.2 per cent of the ad-

* F. A. Loewus and S. Kelly, unpublished observations.

ministered activity in three separate experiments. While incomplete as regards comparison with other labeled constituents within the strawberry, these experiments do provide additional evidence showing that the primary alcohol function of carbon 6 of D-glucose is conserved during the course of its conversion to L-ascorbic acid.

To accommodate the tracer data, a scheme of ascorbic acid synthesis from sugars¹⁹ that differs from the one described by Isherwood *et al.* has been devised. This scheme is outlined in FIGURE 2. Since Hassid *et al.*²⁰ have reviewed their research on the interconversions of sugar nucleotides, we have simplified this scheme by omitting the individual steps leading to D-galacturonic acid and substituting the general term, uridine diphosphate derivatives. Triose

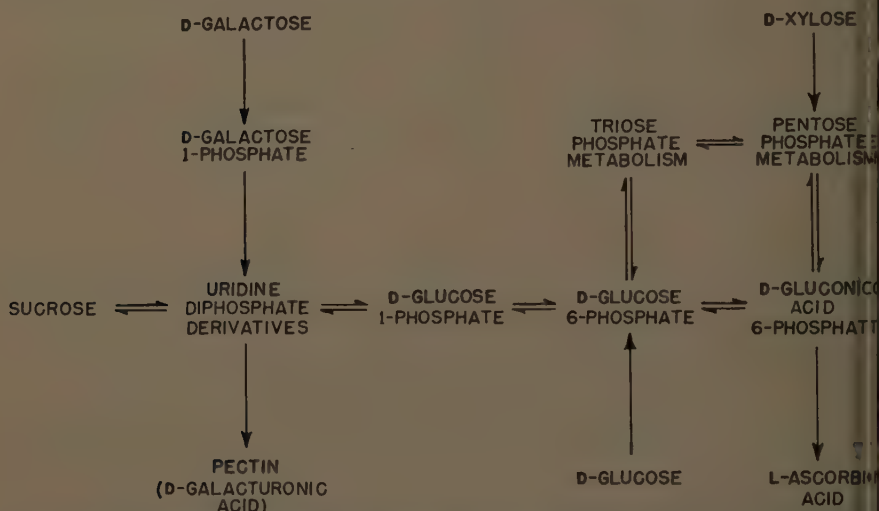


FIGURE 2. A scheme outlining the probable metabolic origin of L-ascorbic acid in relation to sucrose and D-galacturonic acid in plants, based on tracer data obtained from C¹⁴-labeled D-glucose, D-galactose, and D-xylose.

phosphate metabolism, operating reversibly on the pathway from D-glucose to L-ascorbic acid, is primarily responsible for the redistribution of C¹⁴ between opposite halves of the hexose product. There is, however, an additional movement of C¹⁴ from D-glucose-1-C¹⁴ into carbons 2 and 3 of L-ascorbic acid (TABLE 1), probably caused by contributions from pentose phosphate metabolism intimately related to the direct oxidation pathway. Experiments in which D-xylose-1-C¹⁴ and L-arabinose-1-C¹⁴ were administered to ripening strawberries also caused a very considerable redistribution of C¹⁴ between carbons 1, 2, and 3 of L-ascorbic acid (and accounted for 70 per cent of the total C¹⁴ in the molecule).²¹

It has already been pointed out that the more extensive redistribution of C¹⁴ in the ascorbic acid from D-galactose-1-C¹⁴-labeled berries was due to the long pathway of incorporation and greater opportunity for equilibration with triose phosphate as compared to D-glucose-1-C¹⁴. This observation was just

the reverse of that found in rats,²² in which galactose was a more efficient precursor of ascorbic acid than glucose and did not undergo as great a redistribution of C¹⁴. Apparently only one route of ascorbic acid synthesis exists in the plant.²³ This route passes through the uridine diphosphate (UDP) derivatives to UDP-D-glucuronic acid and is more immediately available to D-galactose, which can enter the sugar nucleotide pool directly as galactose-1-phosphate, than it is available to D-glucose, which must first be converted from its 6-phosphate to the 1-phosphate.

The C¹⁴-labeled sugar experiments led to the suggestion^{11,19} that perhaps an oxidation product of D-glucose-6-phosphate is the key intermediate that leads to L-ascorbic acid formation in plants. The fact that it was carbon 1 of D-glucose that was oxidized while carbon 6 remained a primary alcohol function,

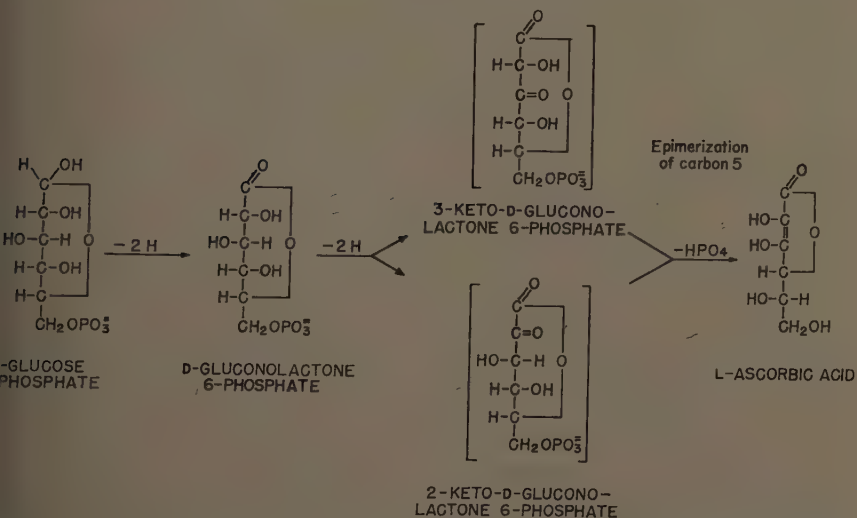


FIGURE 3. A hypothetical scheme for the oxidation of D-glucose-6-phosphate to L-ascorbic acid.

and the parallel appearance of C¹⁴ redistribution patterns in L-ascorbic acid and other products of hexose metabolism, prompted consideration of the direct oxidation pathway of D-glucose as the route of L-ascorbic acid synthesis. These views are still tentative, but a brief consideration of the steps leading to L-ascorbic acid might be in order.

Any consideration of the conversion of D-glucose to L-ascorbic acid that does not involve an inversion of the carbon chain must consider: (1) the oxidation of carbon 1 to a carboxyl group; (2) oxidation of carbon 2 or carbon 3 to a keto group that can eventually form an enediol; and (3) the epimerization of carbon 5 from the D to the L configuration. Evidence for the first consideration is obtained in the tracer data from the experiments with D-glucose-1-C¹⁴. The oxidation of D-glucose-6-phosphate to D-gluconic acid-6-phosphate is well established and might be the first step in the conversion (FIGURE 3). If an oxidation at carbon 2 or 3 follows the oxidation of carbon 1, then the second

requirement could be the same or similar to the second step in the direct oxidation pathway in which D-gluconic acid-6-phosphate is oxidized. Horecker²⁴ has proposed the 3-keto acid as a hypothetical intermediate in the oxidative decarboxylation to D-ribulose-5-phosphate. However, both the 2-keto and the 3-keto acids would yield the same α,β -enediol upon enolization. Perhaps both keto acids are formed, one decarboxylating to give the pentulose, the other stabilizing the carboxyl function through the formation of an enediol.

The third consideration, the epimerization about carbon 5 is, by far, the most ambiguous. The possibility that such a process did occur was quite clear 4 years ago,¹¹ but its enzymatic nature must still be sought. L-Idose-6-C¹⁴ is a very poor source of C¹⁴ for ascorbic acid synthesis in strawberries,²⁵ and it does not appear from this study that an epimerization occurs prior to the formation of D-glucose-6-phosphate. Since the first consideration of the epimerization,¹¹ three separate examples reported from six different laboratories have been found with labeled sugars. It has been found that D-glucose is converted to L-fucose,²⁶⁻²⁸ L-rhamnose,^{29,30} and L-iduronic acid³¹ with the carbon chain intact and an epimerization at carbon 5. One might anticipate the same sort of relationship between the sugar precursor of alginic acid and its uronic constituents, D-mannuronic acid and L-guluronic acid, which differ only about carbon 5.³² It appears likely that a sugar nucleotide is involved in these epimerizations, but whether this also applies to L-ascorbic acid is not known.

Aspects with C¹⁴-Labeled D-Glucuronic Acid

These studies with labeled sugars did not provide an explanation of Isherwood *et al.*'s results, nor did they resemble the results obtained in animal tissues. It was decided to proceed a step further and to test D-glucuronic acid, which had been shown to function as a precursor of ascorbic acid in plants⁴ and animals.⁸ The experimental procedure was the same as it had been with labeled sugars. D-Glucuronate-6-C¹⁴ and its lactone were used in the first attempts.^{11,19} Both compounds were metabolized by the strawberry, and a considerable portion of the C¹⁴ appeared as C¹⁴O₂. Both the ethanol-soluble and ethanol-insoluble fractions were radioactive. The pectinase hydrolyzates of the latter contained radioactive D-galacturonic acid that, upon degradation, was shown to have all of its C¹⁴ in carbon 6.

The activity in the ethanol-soluble portion of the berry resided principally in the acidic constituents. Gradient elution of these acids from Dowex 11 (formate) resin with a 0 \rightarrow 3 N formic acid gradient gave three distinct radioactive peaks, one of which appeared in the region that characterized ascorbic acid. However, repeated recrystallizations of the ascorbic acid after dilution with carrier resulted in a continuous loss of activity. A constant specific activity had not been reached after seven recrystallizations, at which point the yield had dropped to such a low value as to prevent further purification. At the time it was thought the contaminating substance was D-glucuronic acid-6-C¹⁴, since periodate released considerable C¹⁴O₂ from the threonic acid fragment that was supposed to represent only carbons 3 through 6 of ascorbic acid. Subsequent work has shown that a mixture of six-carbon sugar acids was involved.

The considerable loss of activity as $C^{14}O_2$, which was observed when D-glucuronic acid-6- C^{14} was administered to strawberries, prompted experiments in which C^{14} was located in a less labile carbon of the uronic acid. Loewus *et al.*¹² were able to prepare a D-glucuronolactone-1- C^{14} from D-glucose-1- C^{14} of high specific activity without further dilution, by employing paper chromatography at each chemical step in the oxidation procedure. When this compound was metabolized by the strawberry, a distribution of C^{14} different from that found with D-glucuronic acid-6- C^{14} or its lactone was observed. The respired $C^{14}O_2$ was less, the incorporation of C^{14} into the organic constituents was greater, and C^{14} appeared in both the acidic and neutral (sugar) components of the berry.

By modifying the method used to separate the organic acids, a reasonably good separation of the large radioactive peak that had formerly obscured ascorbic acid from ascorbic acid itself was achieved. This was accomplished by using a $0 \rightarrow 0.06 N$ (and sometimes a $0.02 \rightarrow 0.06 N$) formic acid gradient instead of the $0 \rightarrow 3 N$ formic acid gradient used hitherto for elution of organic acids from the Dowex 1 (formate). Thus the incorporation of C^{14} from D-glucuronolactone-1- C^{14} into L-ascorbic acid could be clearly demonstrated. The C^{14} was incorporated almost exclusively into carbon 6, a result that was anticipated by the works of King and of Isherwood, Chen, and Mapson.

The discovery that an over-all path of conversion from D-glucuronic acid to ascorbic acid could be demonstrated with C^{14} in the strawberry, and the obvious differences in C^{14} distribution from D-glucuronolactone labeled at opposite terminal carbons, warranted a more careful study. To this end a comparative study of D-glucuronolactone-1- C^{14} and -6- C^{14} in the ripening strawberry³³ was undertaken. The lactones were carefully purified by paper chromatography immediately prior to use. Both experiments were run simultaneously on berries of comparable size and maturity. The respired CO_2 was collected continuously and assayed periodically for radioactivity. FIGURE 4 shows a plot of the $C^{14}O_2$ accumulation that was almost linear over the 41-hour collection period for the D-glucuronolactone-6- C^{14} -labeled berry but that was sigmoid for the D-glucuronolactone-1- C^{14} -labeled berry. The latter resembled curves obtained with D-glucose-1- C^{14} - and D-xylose-1- C^{14} -labeled berries.

The C^{14} distribution at the end of 41 hours of respiration is summarized in TABLE 4. These values include the activity found in the hulls (sepal, calyx, and stem) and achenes (seeds). Only the achene-free, hull-less, fleshy receptacle was used in subsequent fractionation steps. These steps yielded the C^{14} distribution given in TABLE 5. The activities given in TABLE 5 represent those of the crude fractions prior to isolation and purification of their major constituents. D-Glucuronolactone-1- C^{14} labeled both the acidic and neutral (sugar) constituents, while D-glucuronolactone-6- C^{14} labeled only acidic products. The ethanol-soluble fraction of the latter lost a considerably greater amount of C^{14} (presumably as $C^{14}O_2$) from the Dowex 1 (formate) resin, suggesting the presence of C^{14} -labeled carboxyl functions in labile positions on certain organic acids that were easily decarboxylated under acid conditions. The neutral, non-sugar, fractions of the ethanol-solubles from both berries consisted principally of lactonized sugar acids.

FIGURES 5 and 6 are plots of the radioactivity of Dowex 1 (formate) eluates from the ethanol-soluble and pectinase-hydrolyzed ethanol-insoluble fractions, respectively. Only minor differences were noted in the C^{14} distribution of the acidic components of these fractions when the label originated in carbon 1 rather than carbon 6 of D-glucuronolactone. Peak B (in FIGURE 5) contained the acids that had previously prevented a clean separation of ascorbic acid

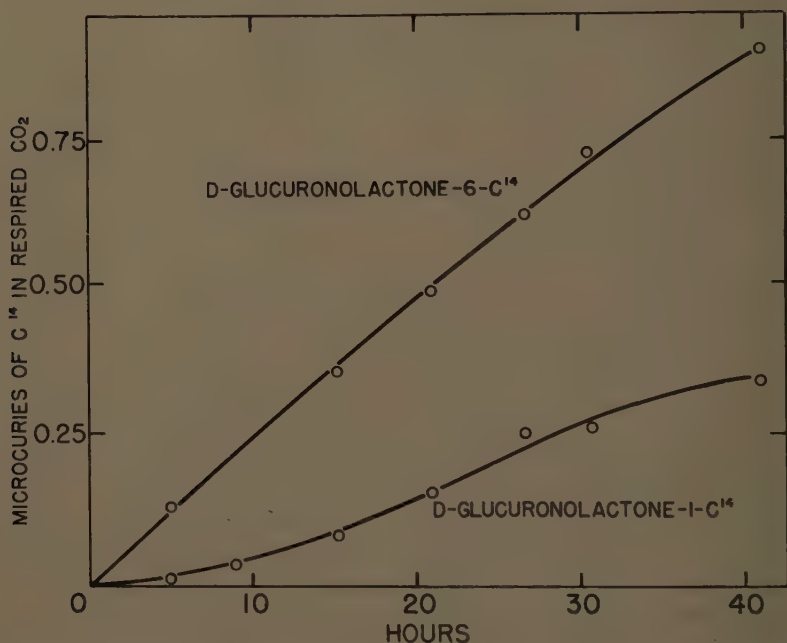


FIGURE 4. A plot of the C^{14} accumulation in the respired CO_2 as a function of time after placing the severed stems of individual ripening strawberries in labeled glucuronolactone. The C^{14} values in the lower plot have been reduced by a factor of 0.76 in order to equate them with the upper plot in terms of administered C^{14} and to permit direct comparison of respired $C^{14}O_2$. Reproduced by permission of the Elsevier Publishing Company, Amsterdam, The Netherlands.³³

TABLE 4
DISTRIBUTION OF RADIOACTIVITY IN D-GLUCURONOLACTONE-1- C^{14} AND -6- C^{14} -
LABELED STRAWBERRIES*

	Site of radioactivity in glucuronolactone	
	Carbon 1	Carbon 6
C^{14} administered, μc .	3.8	2.9
C^{14} recovered, μc .		
Respired CO_2	0.4	0.9
Ethanol-soluble portion	1.7	1.4
Ethanol-insoluble portion	1.7	0.6

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using a more concentrated formic acid gradient. When peak B was chromatographed on paper, seven radioactive components were detected, of which three were very active. Two of these were positively identified as L-gulonic acid and its γ -lactone.³⁴ The third has not been thoroughly characterized but, from its position on the chromatogram relative to L-gulonic acid and its behavior on the ion exchange column, it appears to be gluconic acid. Kessler *et al.*³⁵ have reported that gluconic acid and also glucaric acid are products of D-glucuronic acid metabolism in *Phaseolus aureus* seedlings. Peak C contained ascorbic acid, as indicated by the 2,6-dichlorophenolindophenol titration plot in the upper portion of each graph in FIGURE 5. Other unidentified acids were also present in peak C, but were removed by recrystallizing the ascorbic acid.

TABLE 5

DISTRIBUTION OF RADIOACTIVITY IN THE ETHANOL-SOLUBLE AND PECTINASE-SOLUBILIZED FRACTIONS OF THE RECEPTACLES OF D-GLUCURONOLACTONE-1-C¹⁴- AND -6-C¹⁴-LABELED STRAWBERRIES*

	Site of radioactivity in glucuronolactone	
	Carbon 1	Carbon 6
<i>Ethanol-soluble fraction</i>		
C ¹⁴ added to Dowex 1, μ c.	900	910
C ¹⁴ in L-gulonic acid peak	150	170
in L-ascorbic acid peak	51	34
in other acidic peaks	127	72
in sucrose and xylose	240	Negligible
in other neutral fractions	200	180
lost during separation	120	420
<i>Pectinase-solubilized fraction of ethanol-insoluble residue of berry receptacle</i>		
C ¹⁴ added to Dowex 1, μ c.	194	114
C ¹⁴ in D-galacturonic acid peak	73	80
in other acidic peaks	24	11
in neutral fractions	94	2
lost during separation	3	21

* Reproduced by permission of the Elsevier Publishing Company, Amsterdam, The Netherlands.³³

They accounted for nearly one half of the activity in peak C from the D-glucuronolactone-1-C¹⁴-labeled berry, but were virtually absent in the D-glucuronolactone-6-C¹⁴-labeled berry. Peaks D, E, and F, which were characterized by the regions containing glucuronic acid, malic acid, and citric acid, respectively, have not been carefully studied. In the pectinase-hydrolyzed solubles of the ethanol-insoluble fraction of the receptacles, only D-galacturonic acid gave a prominent radioactive peak (FIGURE 6).

The L-gulonic acid, L-ascorbic acid, and D-galacturonic acid of each berry were isolated, diluted, recrystallized to constant specific activity, and then degraded chemically in order to establish the exact location of the C¹⁴ in the carbon chain of each acid. The results are given in TABLE 6. Very little, if any, redistribution of C¹⁴ was observed in the three different acids. The C¹⁴ appeared in exactly those carbons predicted by the schemes of King⁸ and Isher-

wood *et al.*⁴ Thus D-glucuronolactone-1-C¹⁴ was converted by the strawberry to L-gulonic acid-6-C¹⁴ and L-ascorbic acid-6-C¹⁴, while D-glucuronolactone-6-C¹⁴ was converted to L-gulonic acid-1-C¹⁴ and L-ascorbic acid-1-C¹⁴. These experiments furnish no direct evidence as to whether the L-gulonic acid was

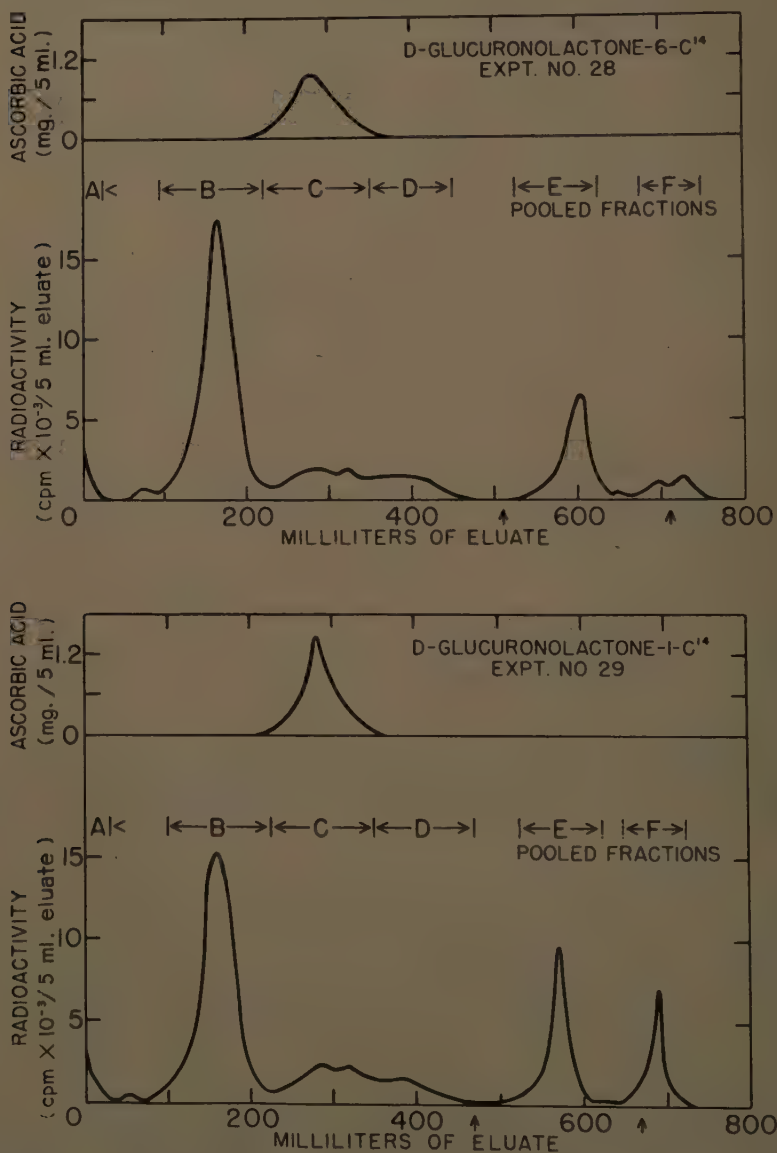


FIGURE 5. Radioactivity and 2,6-dichlorophenolindophenol reducing ability of the fractions (5 ml.) of the ethanol-soluble portion of D-glucuronolactone-1-C¹⁴ and -6-C¹⁴-labeled strawberries, after separation on Dowex 1 (formate) with a 0.02 → 0.06 N formic acid gradient. The arrows below the abscissa indicate the points at which 3 and 6 N formic acid were added to the column.

formed directly from D-glucuronolactone or whether L-gulonolactone was an intermediate in the process. If the latter is true, then the strawberry must contain an active lactonase, since all enzymatic processes were halted by freez-

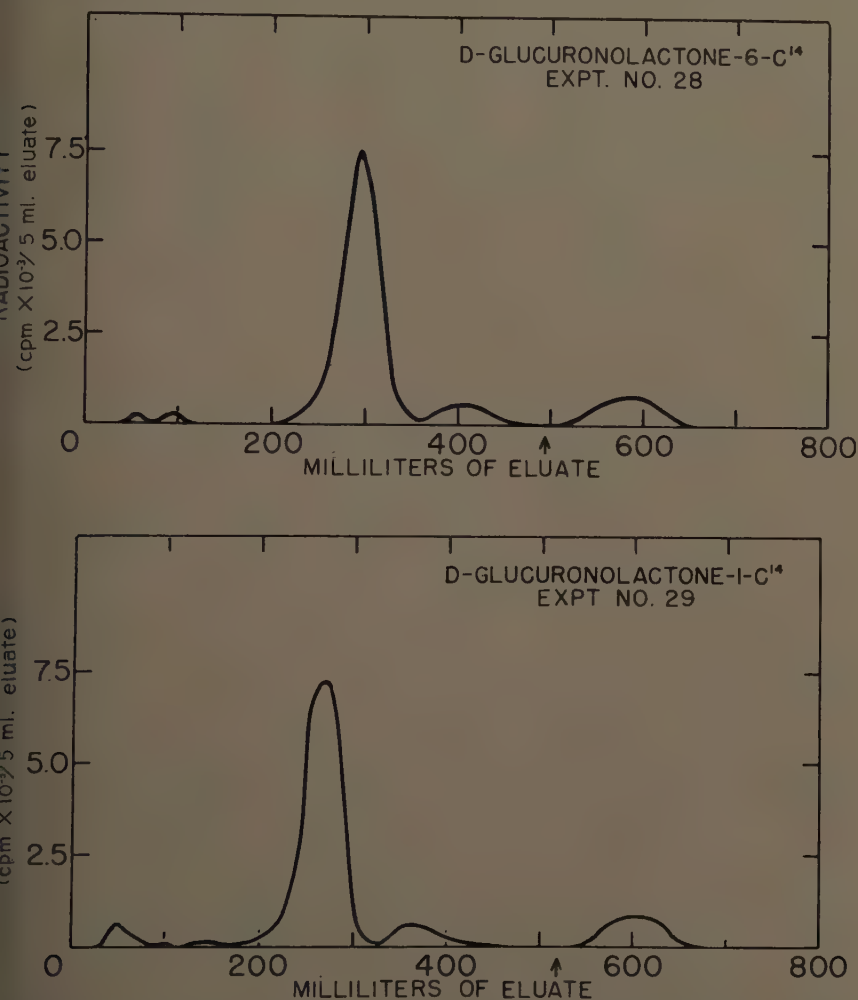


FIGURE 6. Radioactivity of the acidic constituents from the pectinase hydrolyzate of the ethanol-insoluble fraction of D-glucuronolactone-1-C¹⁴- and -6-C¹⁴-labeled strawberries after separation on Dowex 1 (formate) with a 0.02 → 0.06 *N* formic acid gradient. The arrow below the abscissa indicates the point at which 6 *N* formic acid was added to the column.

g the berry prior to fractionation; yet a considerable portion of the L-gulonic acid appeared on the anion column as the free acid. L-Gulonic acid is not a normal constituent of the strawberry, and its occurrence in the present experiments indicates that an enzymatic reduction of D-glucuronic acid as well as galacturonic acid (Mapson and Isherwood³⁶) occurs in plant tissues.

The conversion of D-glucuronolactone-1-C¹⁴ and -6-C¹⁴ to D-galacturonic acid-1-C¹⁴ and -6-C¹⁴, respectively (TABLE 6), by the strawberry amply confirms the original observation¹⁹ made with D-glucuronic acid-6-C¹⁴: D-glucuronic acid is utilized by the plant for pectin synthesis without cleavage of the carbon chain and with only one chemical alteration of the molecule, an epimerization at carbon 4 from the D to the L configuration.

Only when D-glucuronolactone-1-C¹⁴ was administered to the strawberry did appreciable quantities of C¹⁴ reach the sugar constituents. In the ethanol-soluble fraction, this activity was localized in xylose and sucrose.¹² Xylose had up to 20 times as much activity as sucrose, and its specific activity in the same experiment was 15 times greater than sucrose, being about one-third the specific activity of the D-glucuronolactone-1-C¹⁴ used to label the berry. Degradation of the xylose with D-xylose-adapted *Lactobacillus plantarum*³⁷ revealed that all of the C¹⁴ was in carbon 1 (TABLE 7). The C¹⁴ distribution in the D-

TABLE 6
DISTRIBUTION OF RADIOACTIVITY WITHIN THE ACIDIC CONSTITUENTS RECOVERED FROM D-GLUCURONOLACTONE-1-C¹⁴- AND -6-C¹⁴-LABELED STRAWBERRIES

	Site of radioactivity in glucuronolactone					
	Carbon 1			Carbon 6		
	Percentage of C ¹⁴ administered to berry	Percentage of C ¹⁴ incorporated into acid		Percentage of C ¹⁴ administered to berry	Percentage of C ¹⁴ incorporated into acid	
		Carbon 1	Carbon 6		Carbon 1	Carbon 6
D-Galacturonic acid	2	99	1	2.5	—	99
L-Gulonic acid	Approx. 5	—	100	Approx. 5	100	—
L-Ascorbic acid	0.8	—	100	1.0	95	2

xylose and L-arabinose obtained from the pectinase hydrolyzate of the ethanol-insoluble fraction of the berry was limited primarily to carbon 1, but the amount and specific activity of the C¹⁴ in these pentose residues were much lower than the free xylose.

Degradation of the D-glucose moiety of sucrose with *Leuconostoc mesenteroides*³⁷ revealed a rather complex distribution of C¹⁴ (TABLE 7), in which about 90 per cent of the C¹⁴ was localized in carbons 1, 6, 3, and 4, in that order of decreasing activity. This pattern resembles that of hexose monophosphate recovered from pea leaf preparations that had been incubated with ribose-1-phosphate-1-C¹⁴ (Gibbs and Horecker³⁸) and is most conveniently explained on the basis of their observations. The xylose-1-C¹⁴ generated by the D-glucuronolactone, *in situ*, entered the pentose phosphate metabolism of the berry and was converted to hexose phosphate with a C¹⁴ distribution between carbons 1 and 3, but principally in carbon 1. Partial equilibration with triose phosphate resulted in a further distribution of label from carbons 1 and 3 into carbons 6 and 4 respectively. Recent studies by Pubols and Axelrod,³⁹ which demonstrated the isomerization of D-xylose to D-xylulose and inferred the pres-

ence of a xylulose kinase in corn pollen homogenates, provide evidence that a pathway does exist in plant material for the direct entry of D-xylulose or D-xylulose into pentose metabolism. Studies of D-xylulose-1-C¹⁴ metabolism in the strawberry²¹ led to similar conclusions.

Evidence that free xylose recovered from D-glucuronolactone-1-C¹⁴ is of the D configuration rests upon the following observations. It is readily fermented by D-xylose-adapted *Lactobacillus plantarum*. Successive recrystallization with added nonisotopic D-xylose resulted in no change in the specific activity. When the diluted D-xylose was oxidized to D-xylonic acid or converted to its dibenzilidene dimethyl derivative there was no loss of activity. Finally, the fact that the strawberry can utilize this pentose *in situ* for the synthesis of sucrose is positive evidence in favor of the D-configuration.

TABLE 7

DISTRIBUTION OF RADIOACTIVITY WITHIN THE SUGAR CONSTITUENTS RECOVERED FROM D-GLUCURONOLACTONE-1-C¹⁴-LABELED STRAWBERRIES

Sugar	Experiment*	Percentage of C ¹⁴ administered to berry†	Percentage of C ¹⁴ incorporated into each carbon atom					
			C-1	C-2	C-3	C-4	C-5	C-6
Free D-xylose	2	8.1	99	—	—	—	—	—
	29	3.8	84	11	11	11	5	—
Pectinase-hydrolyzed D-xylose	2	0.5	92	8	8	8	8	—
	29	0.3	90	8	8	8	2	—
Pectinase-hydrolyzed L-arabinose	2	0.5	100	—	—	—	—	—
	29	1.0	99	—	—	—	—	—
Glucose moiety of sucrose	2	0.2	42	4	13	12	5	24
	29	0.5	49	4	15	10	2	20

* Experiment No. 2 metabolized 66 hours. Experiment No. 29 metabolized 41 hours.

† These values represent the C¹⁴ actually recovered in the final crystalline products and, therefore, minimal figures for comparative purposes only.

Aspects with D-Galacturonic Acid-1-C¹⁴

The D-glucuronolactone-1-C¹⁴ experiments provided an opportunity to extend the observations to D-galacturonic acid-1-C¹⁴, since the latter is formed from the former in the strawberry and converted directly into pectin with essentially no redistribution of label. Careful hydrolysis of the pectin of a D-glucuronolactone-1-C¹⁴-labeled berry and subsequent recovery with no dilution, beyond that which occurred endogenously, resulted in a quantity of D-galacturonic acid-1-C¹⁴ sufficient for one experiment. It was administered through the severed stems of three strawberries over a period of 94 hours. The uptake of label was very poor, as would be expected of an acid-salt mixture (the label was given as the sodium salt at pH 3 to 4), but more applicable than an experiment involving the use of a derivative such as the methyl ester. Sufficient C¹⁴ did reach the receptacles to permit recovery and degradation of L-ascorbic acid and D-galacturonic acid (from the 70 per cent ethanol-insoluble residue)

as well as autoradiography of the neutral fraction from the Dowex 1 (formate) resin after separation on paper chromatograms.

The results paralleled those obtained with D-glucuronolactone-1-C¹⁴. A very radioactive acid peak preceded that of ascorbic acid when the Dowex 1 resin, containing the ethanol-soluble acids, was eluted with a dilute formic acid gradient. The characterization of this peak has not been completed but, in analogy to the L-gulonic acid, it will probably be L-galactonic acid. The ascorbic acid was recovered and then converted to the dianil derivative of dehydroascorbic acid⁴⁰ to permit recrystallization from aqueous solution and to insure the purity of the product before chemical degradation. Most of the C¹⁴ was found in carbon 6 (TABLE 8). This finding constitutes the first tracer evidence for an over-all conversion of D-galacturonic acid to L-ascorbic acid and neatly demonstrates the "inversion" of the carbon chain predicted by Isherwood *et al.*⁴ six years ago.

The ethanol-insoluble residue of the berries that contained the pectin was thoroughly washed with 70 per cent ethanol until the wash was free of radio-

TABLE 8
DISTRIBUTION OF C¹⁴ IN ACIDIC CONSTITUENTS OF STRAWBERRIES
LABELED WITH D-GALACTURONIC ACID-1-C¹⁴ *

Acidic constituent	Undiluted specific activity (cpm/mg. carbon)	Percentage of C ¹⁴ incorporated into each carbon atom	
		Carbon 1	Carbon 6
Pectinase-hydrolyzed D-galacturonic acid	3200	99	1
L-Ascorbic acid	2100	—	93

* F. A. Loewus and S. Kelly, unpublished data.

activity and then was hydrolyzed with fungal pectinase. The D-galacturonic acid that was recovered had all of its activity in carbon 1 (TABLE 8). Thus D-galacturonic acid, as well as D-glucuronic acid, can supply the uronic acid precursor of pectin. Hassid *et al.*²⁰ have demonstrated the presence of a glucuronic acid kinase and the subsequent steps leading to UDP-D-galacturonic acid in *Phaseolus* preparations. Apparently, the same or a similar kinase is responsible for the incorporation of D-galacturonic acid into pectin shown in the present experiment and by the recent work of Kessler *et al.*³⁵

Although the activity of the neutral ethanol-soluble fraction was quite low it was chromatographed on paper and stored against X-ray film in a cassette for 30 days. At the end of this period a single dark band was visible on the film in the region that characterized xylose on the paper. There was no darkening in the arabinose region. A previous experiment with an L-arabinose-labeled strawberry²¹ showed that this sugar is converted to free D-xylose-1-C¹⁴ prior to further metabolism by the berry. In the present experiment there is no possibility of deciding the exact origin of the xylose: whether it comes directly from D-galacturonic acid via the sugar nucleotides,⁴¹ indirectly via an oxidative decarboxylation followed by a reduction, or indirectly via a decarboxylation

followed by an epimerization of the free pentose. Only further study will clarify this interesting observation.

Aspects of Uronic Acid Metabolism in Plants

Indications of at least two separate pathways of uronic acid metabolism in plants can be seen from the tracer experiments. One, involving uridine diphosphate (UDP) interconversions, as described by Hassid *et al.*,²⁰ eventually leads to the formation of pectin, pentosans, acidic gums, and related plant products. The other, presumably responsible for ascorbic acid synthesis from uronic acids, does not appear to involve UDP intermediates, although the participation of other nucleotides is not excluded and even the lack of a relationship to UDP is not well established.

Concerning the UDP pathway, UDP-D-glucose is the parent UDP sugar in the intact plant. Oxidation of this sugar nucleotide by UDP-D-glucose dehydrogenase leads to UDP-D-glucuronic acid. Strominger and Mapson have purified and characterized this dehydrogenase from pea seedlings.⁴² UDP-D-glucuronic acid can be converted by plant enzymes to UDP-D-galacturonic acid, UDP-D-xylose, and UDP-L-arabinose.²⁰ When D-glucuronic acid or galacturonic acid is administered to a plant from an external source, it is phosphorylated and then converted to its UDP-derivative, which can, apparently, participate only in nucleotide reactions leading toward polysaccharide formation and related energy-conserving or energy-yielding processes. The tracer data reveal little or no redistribution of C¹⁴ within the carbon chain during the conversion of uronic acids to pectin or pentosans in the strawberry, suggesting that there is not a close metabolic interchange between the hexose phosphate pool and the already-formed UDP-uronic acids, except by re-entry of free xylose generated by the decarboxylation of uronic acid. The evidence that the latter might be related to UDP processes rather than the second pathway (see below) must still be obtained. All of the preceding discussion should be tempered by the statement that the observations have been limited to the ripening process in the strawberry, and experiments to establish their general nature should be performed.

The second uronic acid pathway was first described by Horowitz and King⁸ for D-glucuronic acid, and by Isherwood *et al.*⁴ for D-galacturonic acid. Tracer experiments have revealed that both of these uronic acids are converted to ascorbic acid by the strawberry. Neither Horowitz and King's nor Isherwood's scheme of L-ascorbic acid biosynthesis could be extended, in plant tissues, to include the parent sugars of these uronic acids, which are apparently stabilized to L-ascorbic acid over quite a different pathway. The discovery of L-gulonic acid in strawberries labeled with D-glucuronolactone supports the view that the uronic acid is first reduced to an aldonic acid followed by an oxidation to L-ascorbic acid.

A Proposed Scheme Common to Both the Sugar and Uronic Acid Pathways to Ascorbic Acid

One might conceive of an isomerization from an aldo-uronic acid to a keto-uronic acid such as that described by Ashwell *et al.*⁴³ in bacterial systems.

Stereospecific reduction of D-fructuronic acid, the keto-uronic acid derived from D-glucuronic acid, would lead to either L-gulonic or D-mannonic acid, while D-tagaturonic acid, from D-galacturonic acid, would yield L-galactonic or D-altronic acid. It is very possible that the enzyme responsible for the reduction of keto-uronic acids is the same as the one that reduces ketoses to polyols. The specificity of the plant polyol dehydrogenases differ from those found in the rat (Edson⁴⁴), and there is evidence of two polyol dehydrogenases in *Sorbus aucuparia* with specificities for opposite penultimate carbons of D-sorbitol

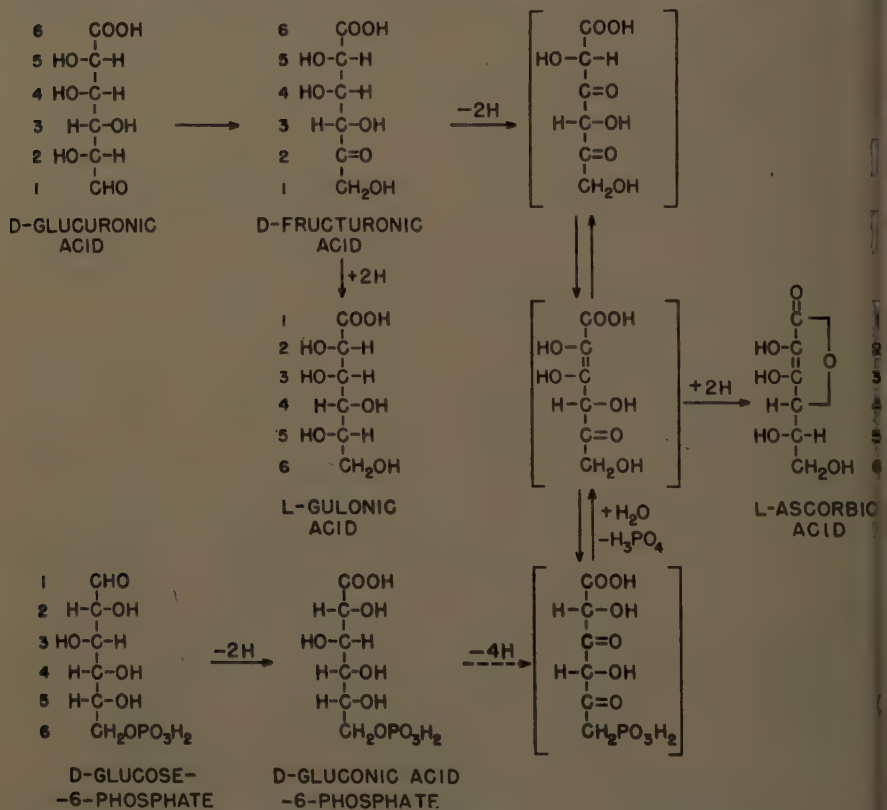


FIGURE 7. A hypothetical scheme for the synthesis of L-ascorbic acid and L-gulonic acid from D-glucuronic acid and for the synthesis of L-ascorbic acid from D-glucose-6-phosphate.

(McCorkindale⁴⁶). If the enzyme that Edson refers to as L-iditol dehydrogenase also reduced D-fructuronic acid, the product would be L-gulonic acid. The formation of a free aldonic acid rather than its lactone complicates any proposed scheme of L-ascorbic acid synthesis, however, since most enzymatic studies have demonstrated a need for the lactone.⁴⁶⁻⁵¹ If the occurrence of free D-xylose-1-C¹⁴ from D-glucuronolactone-1-C¹⁴ could also be shown to be associated with the ascorbic acid-producing pathway of uronic acid metabolism, the tracer data would be simpler to interpret.

A hypothetical scheme that accommodates the tracer results and avoids some of the problems mentioned already is diagrammed in FIGURE 7. No claim is

made for this scheme other than its value to stimulate consideration of an intermediate common to both the "uronic acid to ascorbic acid" and the "hexose to ascorbic acid" pathways. The first step would be the isomerization of the uronic acid to a keto-uronic acid. This could be either reduced or oxidized. If it were reduced, the product would accumulate as L-gulonic or L-galactonic acid. If it were oxidized, the resulting diketo acid would be an intermediate with properties common to both pathways. The assumption has been made here that the oxidation would occur α or β to the carboxyl function in analogy to the enzyme studies in animal systems. Only the β -oxidation is shown in FIGURE 7, but similar considerations would apply if the oxidation were α . Enolization of the keto function between the α and β carbons would stabilize the carboxyl group and leave the 5-keto group exposed to undergo a stereospecific reduction (L-iditol dehydrogenase²⁴) to L-ascorbic acid. Such a diketo acid might also be the precursor of free D-xylose, since a β -decarboxylation followed by a stereospecific reduction would form D-xylulose directly.

There are other attractive features to be considered in this diketo acid intermediate. Since enolization would destroy the stereochemistry of the α and β carbons, D-gluconic acid derivatives could, upon oxidation, arrive at the same diketo acid structure. This is shown with the oxidation of D-glucose-6-phosphate at the bottom portion of FIGURE 7. It would also explain the presence of free gluconic acid as a product of D-glucuronic acid metabolism in *Phaseolus* seedlings.³⁵ Although the tracer results favor such a scheme, they do not exclude others. Only patient and careful study of the experimental details that have been uncovered by these exploratory experiments with C¹⁴ will reveal the full nature of the synthetic pathway.

Conclusions

The isotopic studies have provided evidence of two separate pathways of ascorbic acid formation in higher plants. The pathway that plays the normal biosynthetic role in intact plant tissues must still be sought experimentally. The present view of my colleagues and of myself favors the process that can be demonstrated with C¹⁴-labeled D-glucose and D-galactose. If it is assumed that sucrose is the major carbohydrate of translocation in the plant and the major source of carbon for hexose phosphate synthesis, then it is probably the true "parent" carbohydrate precursor of L-ascorbic acid. The "sugar to ascorbic acid" pathway appears to follow the same enzymatic steps that describe the direct oxidation of hexose monophosphate, but a mechanism for the change from D to L configuration about carbon 5 of the hexose must still be sought.

The enzymatic aspects of the "uronic acid to ascorbic acid" pathway have been studied in great detail by other investigators, but the physiological significance of these interconversions is by no means clear. Uronic acids are seldom found in the free state in intact plants. Their appearance is usually associated with pathological disturbances, physiological changes such as senescence, and various types of physical damage (cutting or crushing of the tissue). Such conditions could lead to the release of hydrolytic enzymes that would convert plant cell-wall polysaccharides to the free sugars and uronic

acids; the latter could then undergo the synthetic steps leading to ascorbic acid. Measurable increases have been observed in potato tubers and apples after their tissues were cut and exposed to the air for several hours.^{3,52,53} Study of the causal relation between ascorbic acid formation and tissue damage observed in these studies may shed some light on the significance of the uronic acid pathway.

Summary

C¹⁴-labeled sugars and uronic acids have been used to explore the conversion of D-glucose, D-galactose, D-glucuronolactone, and D-galacturonic acid to L-ascorbic acid in the detached ripening strawberry fruit (*Fragaria*). These precursors, containing C¹⁴ at a specific carbon atom, were administered by injection or by uptake through the cut stem of individual berries. After a period of metabolism, the component sugars, organic acids, and polysaccharides of each berry were isolated and degraded to determine the specific location of C¹⁴ in L-ascorbic acid, sucrose-derived D-glucose, free D-glucose, free D-xylose, and pectin-derived D-galacturonic acid, D-xylose, and L-arabinose. The results reveal the presence of two different pathways to L-ascorbic acid synthesis in the strawberry. One, traced from D-glucose and D-galactose, proceeds through hexose phosphate (including some equilibration with triose phosphate) and results in L-ascorbic acid with the same carbon sequence as the parent sugar; that is, D-glucose-1-C¹⁴, -2-C¹⁴, or -6-C¹⁴ is converted to L-ascorbic acid labeled principally in carbons 1, 2, or 6 respectively. The other pathway, traced from D-glucuronolactone and D-galacturonic acid, proceeds over a pathway that does not, apparently, involve phosphorylated intermediates and is converted to L-ascorbic acid with an inverted carbon sequence compared to the parent uronic acid; that is, D-glucuronolactone-1-C¹⁴ or D-galacturonic acid is converted to L-ascorbic acid-6-C¹⁴. The possible nature of these interconversions is discussed.

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THE INTERRELATIONSHIP BETWEEN GULONOLACTONE OXIDASE AND THE PROCESS OF LIPID PEROXIDATION

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In 1958 our group reported that the synthesis of ascorbic acid by liver homogenates obtained from animals deprived of vitamin E is impaired when glucuronic acid is used as a starting substrate.¹ The impairment was subsequently found to be due to the inhibition of the microsomal enzyme, gulonolactone oxidase.² The reduction of glucuronate to gulonate or the formation of gulonolactone from gulonate are normal or perhaps slightly increased.

Solubilization and Studies of Some Properties of Gulonolactone Oxidase

In order to study the mechanism of the inhibition of gulonolactone oxidase, it appeared that it would be useful to purify the enzyme and study its properties but, unfortunately, our efforts in this direction have not been very rewarding. We solubilized the enzyme by using sodium cholate.³ As may be seen in TABLE 1, this salt gave better results in our laboratory than those obtained by using deoxycholate, even if the deoxycholate seems to be the one preferred by other groups. It is not our purpose in this paper to make a survey of the properties of gulonolactone oxidase, but we shall mention some aspects especially related to this subject. We reported recently that all the preparations available to us at the time were activated by addition of hydrogen peroxide to higher levels than the activity obtained in 100 per cent oxygen.⁴ Subsequently however, we found preparations that were not activated by H_2O_2 , and this led us to a comparative study of the activating effects of H_2O_2 and O_2 .⁵ The results obtained were rather surprising. FIGURE 1 shows that gulonolactone oxidase requires approximately 10 atmospheres of O_2 in solution for maximum activity and that the activity of catalase on 30 μ moles H_2O_2 in a system open to the air is able to produce O_2 tensions of these magnitudes. The low affinity of gulonolactone oxidase for O_2 may have some relationship with the great sensitivity to the inhibitory action of lipid hydroperoxide reported below. If this were a competitive inhibition mechanism, the low affinity of the enzyme for O_2 would enhance the inhibitory effect.

The Mechanism of the Inactivation of Gulonolactone Oxidase in Vitamin E Deficiency

With respect to the mechanism of inactivation of ascorbic acid synthesis we must declare ourselves fortunate in that we failed in our earliest attempt to activate this system with tocopherol. We obtained reactivation first with such agents as Mn^{++} and Co^{++} ions and ethylenediaminetetraacetic acid (EDTA);¹ this was somewhat disconcerting at that time because these substances were expected to produce effects antagonistic among themselves.

Therefore, when it was found that tocopherol also reactivated, we were restrained from concluding that tocopherol was the coenzyme of gulonolactone oxidase; this, of course, does not mean that tocopherol is not the coenzyme.

The clue that eventually led to the description of the mechanism of the inactivation was disclosed by McCay and Carpenter in our group, who found that during the reaction catalyzed by the extracts of the deficient animals, a sub-

TABLE 1
COMPARISON OF CHOLATE AND DEOXYCHOLATE AS SOLUBILIZER
OF GULONOLACTONE OXIDASE*

Preparation	Ascorbic acid synthesized (μ moles/hour)	Protein content (mg.)	Specific activity (μ moles/hour/mg.)
Cholate-soluble	3.9	27.0	0.142
Cholate-insoluble	0.3	39.3	0.009
Deoxycholate-soluble	3.1	84.0	0.037
Deoxycholate-insoluble	0.3	17.0	0.018

* Conditions and methods according to Kitabchi *et al.*³

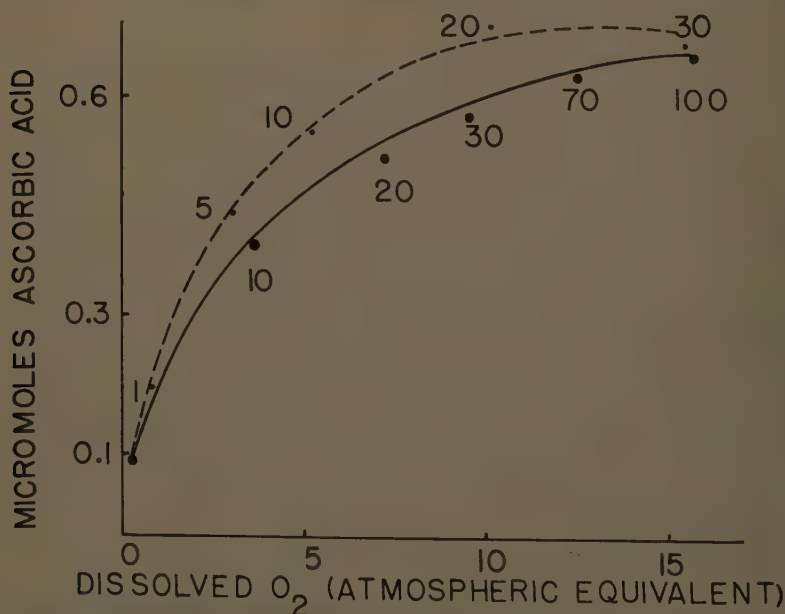


FIGURE 1. Activation of gulonolactone oxidase by O₂ under pressure (---) and by H₂O₂ (—) in the presence of catalase. Numbers above broken line are the atmospheric pressures of pure O₂ to which the solutions were subjected. Numbers below continuous line are the μ moles of H₂O₂ added. Incubation system: rat liver microsome suspension, 0.1 ml.; L-gulonolactone, 10 μ moles; phosphate buffer, pH 7.5 to a final volume of 1 ml.; incubation period, 15 min.; temperature, 25° C. The incubations with H₂O₂ were done in test tubes open to the atmosphere; the incubations with pressurized O₂ were done in 5-ml. beakers inside a specially devised chamber; the substrate was introduced into the system from a separate container by shaking after 2 min. of gassing and 10 min. of equilibration. Reproduced by permission of *Biochemical and Biophysical Research Communications*.

stance was produced that reacted with thiobarbituric acid.⁶ This substance was later identified as malonaldehyde, which usually appears as a product of the process of lipid peroxidation. As may be seen in TABLE 2, all the agents tested that activated gulonolactone oxidase at the same time decreased the formation of malonaldehyde (or thiobarbituric acid-reacting material). A clearer description was obtained after we succeeded in solubilizing and fractionating the gulonolactone oxidase preparations; as a result, the difference between the enzymic activities obtained from tocopherol-sufficient and tocopherol-deficient animals practically disappears.³ If to a cholate-solubilized preparation from microsomes obtained from tocopherol-deficient animals,

TABLE 2
EFFECT OF VARIOUS AGENTS ON SYNTHESIS OF ASCORBIC ACID AND PRODUCTION OF THIOPHORBIC ACID-REACTING MATERIAL BY LIVER PREPARATIONS OF VITAMIN E-DEFICIENT RATS AND THEIR CONTROLS*

Addition to test system†	Supplement to diet	Ascorbic acid synthesis			Thiobarbituric acid		
		No. exps.	No add.	With add.	No. exps.	No add.	With add.
			$\mu\text{moles/gm./2 hours}$			O.D. 535 $m\mu$	
Co ⁺⁺	None	9	0.25	1.71	5	0.257	0.028
	Vitamin E	7	1.47	2.00	3	0.005	0.011
Mn ⁺⁺	None	9	0.28	1.22	4	0.375	0.028
	Vitamin E	7	1.39	1.68	4	0.014	0.000
Ce ⁺⁺⁺	None	6	0.36	1.20	4	0.487	0.060
	Vitamin E	2	1.31	1.02	3	0.000	0.000
Quinone 2 mg./ml.	None	2	0.31	1.00	2	0.281	0.000
	Vitamin E	2	1.19	0.56	2	0.000	0.000
EDTA $6 \times 10^{-6} M$	None	13	0.21	1.36	6	0.258	0.025
	Vitamin E	7	1.53	1.22	4	0.019	0.020

* Conditions and methods according to Carpenter *et al.*⁶

† Except where stated otherwise, the concentration is $5 \times 10^{-4} M$.

Ammonium sulfate is added to a final concentration of 40 per cent saturation, two fractions are obtained that we called 40 fraction (the precipitate) and 90 fraction (the supernate). It may be seen in TABLE 3 that the 40 fraction apparently contains most of the gulonolactone oxidase and that during the incubation of this fraction no malonaldehyde is produced. The 90 fraction, on the other hand, has no gulonolactone oxidase activity but produces malonaldehyde. If we mix both (40 and 90 fractions), the activity is inhibited to a level somewhat less than 50 per cent of the total or about 50 per cent of the activity of the 40 fraction alone. The nature of the inhibitor may be seen in Experiment No. 6 in TABLE 3. The lyophilized 90 fraction was extracted with a chloroform-methanol mixture, and a material was obtained that we designated as lipid. When this lipid was added to the incubation system it was found responsible for the inactivation of the 40 fraction. The last experiment shows again that tocopherol prevents this inhibition. TABLE 4 shows that the 90

fraction is only apparently inactive. If Co^{++} , or Mn^{++} , or EDTA are added to it, the production of malonaldehyde decreases and the synthesis of ascorbic acid increases substantially. It was shown that malonaldehyde by itself is not the inhibitor of gulonolactone oxidase.³

It was fortunate for the detection of the effect of the deficiency of tocopherol on the synthesis of ascorbic acid that the oxidation of gulonolactone to form ascorbic acid is a slow reaction. FIGURE 2 shows the rates of the reactions carried out by the fraction 40 and by fraction 40 plus 90 lipid. It may be seen that both systems have approximately the same activity during the first

TABLE 3
MALONALDEHYDE PRODUCTION AND ASCORBIC ACID SYNTHESIS BY THE 40 AND 90 MICROSOMAL FRACTIONS*

Experiment No.	Fractions	Additions	Ascorbic acid synthesized ($\mu\text{moles}/2$ hours)	Malonaldehyde produced (O.D. 535 $m\mu$)
1	40	—	0.34	0.000
2	90	—	0.05	0.717
3	40 + 90	—	0.17	0.100
4	40 + heated 90	—	0.09	0.830
5	Heated 40 + heated 90	—	0.00	0.018
6	40	90 lipid	0.07	0.747
7	40	90 lipid + tocopherol	0.41	0.005

* Conditions and methods according to Kitabchi *et al.*³

TABLE 4
MALONALDEHYDE PRODUCTION AND ASCORBIC ACID SYNTHESIS BY THE 90 MICROSOMAL FRACTION*

Additions	Ascorbic acid synthesized ($\mu\text{moles}/2$ hours)	Malonaldehyde produced (O.D. 535 $m\mu$)
—	0.12	0.717
Co^{++}	0.37	0.007
Mn^{++}	0.36	0.004
EDTA	0.35	0.006

* Conditions and methods according to Kitabchi *et al.*³

30 min., but the system with 90 lipid becomes almost completely inactive after the first 30 min. In our opinion this is strong support to the hypothesis that the inhibitor is formed at the time when the reaction is being carried out.

Factors Promoting Lipid Peroxidation

At this point our problem was to decide which factors promoted the formation of the inhibitor. TABLE 5 shows that Fe^{++} salts acting on the microsomes produce malonaldehyde; this probably is the principal mechanism of lipid peroxidation in the crude homogenate, consisting of microsomes and supernate, but in the washed microsomes or in the soluble preparations obtained therefrom, there is not enough Fe^{++} salt to catalyze this process efficiently. It has

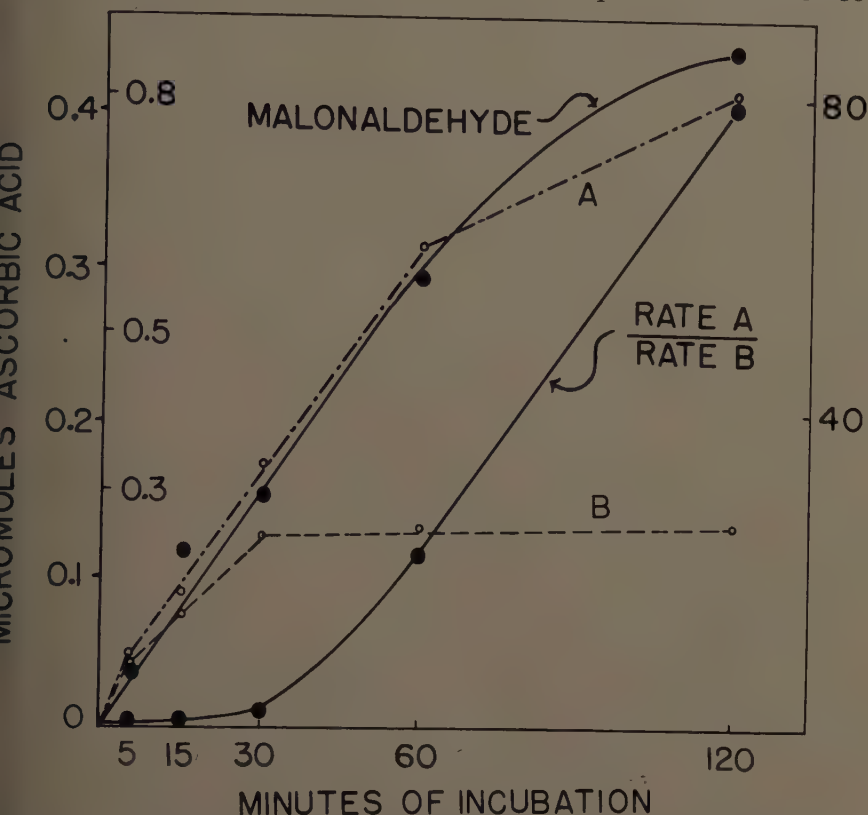


FIGURE 2. Inhibition of gulonolactone oxidase and the formation of malonaldehyde. System A (---○---), ascorbic acid synthesis: fraction 40, 0.05 ml.; gulonolactone, 2 μ moles; 0.15 M phosphate buffer, pH 7.4; total volume 1 ml.; B (---○---) ascorbic acid synthesis: system as A but with the addition of 0.05 ml. of lipid extracted from fraction 90; (—●—) malonaldehyde production by system B expressed as O.D. at 535 $m\mu$ (inside of left ordinate); rate A/rate B (—●—) rate of inhibition of gulonolactone oxidase (right ordinate); each point of this curve is calculated as the ratio of the amount of ascorbic acid synthesized during each time interval in systems A and B. Reproduced by permission of *The Journal of Biological Chemistry*.³

TABLE 5
PRODUCTION OF MALONALDEHYDE FROM MICROSOMAL LIPID OBTAINED FROM VITAMIN E-DEFICIENT RATS*

Additions		No. of experiments	Malonaldehyde produced (O.D. 535 $m\mu$)
Compound	Concentration ($\times 10^5 M$)		
—	—	4	0.069
Supernatant	—	1	0.254
Fe ⁺⁺⁺	2.5	11	0.735
Ascorbic acid	20.0	2	0.414

Conditions and methods according to Kitabchi *et al.*³

been known for some time that ascorbic acid is able to catalyze the peroxidation of fat, and since ascorbic acid is formed here it may initiate a process that eventually stops its own synthesis. There can be little doubt that ascorbic acid does catalyze the process of peroxidation since malonaldehyde was formed when microsomal lipid was incubated with this acid, as may be seen in TABLE 5. The problem is whether ascorbic acid is the only catalyst for the process of lipid peroxidation in the system in which ascorbic acid itself is synthesized.

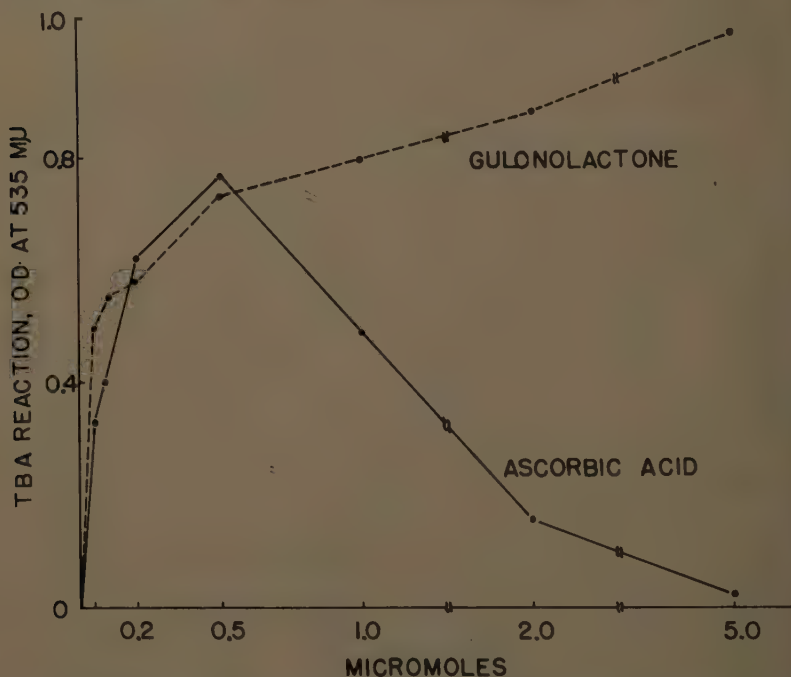


FIGURE 3. Production of malonaldehyde by incubation of vitamin E-deficient microsomes with gulonolactone or ascorbic acid: 0.05 ml. microsomal suspension (equivalent to 50 mg. of liver); amount of L-gulonolactone or L-ascorbic acid varied from a minimum of 0.05 to a maximum of 5 μ moles; 0.15 M phosphate buffer, pH 7.4; total volume, 1 ml.; incubation period, 1 hour; temperature, 37° C.

FIGURE 3 shows one of the experiments done in an attempt to solve this problem.

The formation of malonaldehyde was studied when the enzymic system gulonolactone oxidase was incubated with different amounts of gulonolactone or with ascorbic acid. It may be seen that in the case of ascorbic acid there is an optimum concentration of the acid (at about 0.5 μ mole/ml.) for the production of malonaldehyde; at higher concentrations, ascorbic acid acts as an antioxidant. The observation that at concentrations up to 0.5 μ mole, equal amounts of ascorbic acid and gulonolactone produce equal amounts of malonaldehyde is an indication that there is an added mechanism of lipid peroxidation in the case of the oxidation of gulonolactone because the transformation

of gulonolactone to ascorbic acid is not quantitative. Furthermore, we see in FIGURE 3 that the values of malonaldehyde with gulonolactone can be higher than the values obtained with any concentration of ascorbic acid. Even if, admittedly, the values are not very much higher, we have considered this observation as in favor of the existence of another catalyst besides ascorbic acid in the purified system. These findings are summarized in FIGURE 4.

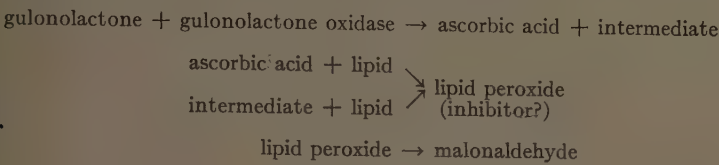


FIGURE 4.

TABLE 6

COMPARISON OF THE ACTIVITIES OF SOME LIVER ENZYMES IN PREPARATIONS FROM VITAMIN E-SUFFICIENT AND VITAMIN E-DEFICIENT RATS

Enzyme	Preparation	No. of experiments	Enzymatic activity	
			Vitamin E-sufficient rats	Vitamin E-deficient rats
gulonolactone oxidase	Microsomes	18	0.229*	0.117*
glutathione S-transferase C	Microsomes	6	0.062†	0.057†
phenylalanine NH oxidase	Microsomes and supernatant	9	0.440‡	0.420‡
glutathione S-transferase	Microsomes and supernatant	6	5.36§	4.50§

* O.D. at 540 mμ; assayed according to Kitabchi *et al.*³
† O.D. at 500 mμ; assayed according to Roy.⁷
‡ Δ O.D. at 340 mμ/min.; assayed according to Strittmatter, *et al.*⁸
§ Milliequivalents of perborate consumed; assayed according to Feinstein.⁹

Specificity of the Inhibitory Effect of Lipid Peroxide on Gulonolactone Oxidase

The reason for the sensitivity of gulonolactone oxidase to the process of lipid peroxidation has not yet been found. Attempts were made to extend these findings to other enzymes, but we have failed. The following systems have been tested: mitochondrial oxidation, oxidative phosphorylation, phosphogluco-6-phosphate dehydrogenase, D-amino acid oxidase, phosphatases, dipeptidase, and other enzymes shown in TABLE 6. We studied these with especial persistence not only because they were microsomal enzymes (as gulonolactone oxidase), but also because some of them were SH and flavin enzymes. In the case of D-amino acid oxidase, a decrease of the activity in the preparations from tocopherol-deficient animals was observed, but the statistical analysis showed that the difference was not significant. We call attention to the failure with these enzymes because it is generally accepted that peroxides will inactivate SH enzymes. If in the case of gulonolactone oxidase, the mechanism is through its action on the SH groups, we should have to assume that its SH groups are

especially exposed to attack by lipid peroxides. We believe it is worth noting that gulonolactone oxidase is very easily inactivated by lipid peroxide but is very resistant to the actions of O_2 at high pressures.

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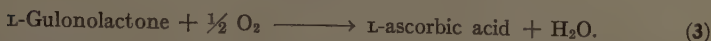
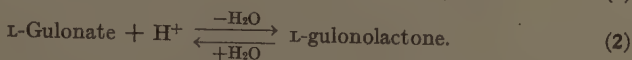
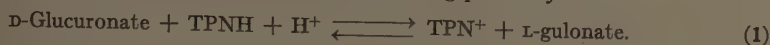
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THE ROLE OF ALDONOLACTONASE IN THE BIOSYNTHESIS OF L-ASCORBIC ACID*

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Work in several laboratories¹⁻⁶ has shown that D-glucuronate is converted into L-ascorbate in animal tissues by the following pathway:



The three enzymes that catalyze the reactions shown above have been separated from one another and their properties studied.

Our detailed studies of purified pig kidney TPN-L-gulonate dehydrogenase,⁷ which catalyzes Reaction 1, have shown that the enzyme reduces only the glucuronate anion but does not reduce glucuronolactone or UDP-glucuronate. On the other hand, Mano *et al.*⁸ claim that the liver dehydrogenase reduces glucuronolactone more readily than glucuronate even though the relative rates of oxidation of gulonate and gulonolactone by the two enzymes are identical. These differences appear to be due probably to an actual difference in specificity of the two enzymes, depending on the source, the manner in which the gulonates used as substrates were prepared from glucuronolactone, or on the ratio of the rates of the nonenzymatic hydrolysis of the lactone to the rate of the dehydrogenase reaction, and to the *pH*. The failure of the enzyme to reduce UDP-glucuronate as well as other lines of evidence, such as the inability of preformed glucuronolactone or glucuronate to give rise to glucuronides in intact animals,⁹⁻¹¹ the failure to detect the UDP-glucuronate pyrophosphorylase reaction in animal tissues,¹² and the finding that in intact animals glucuronolactone is a more efficient precursor of ascorbate than glucose,¹³ exclude a pathway of ascorbate formation from UDP-glucuronate involving uridine nucleotide derivatives.

Washed rat liver microsomes contain L-gulonolactone oxidase, which catalyzes Reaction 3, as first noted by Burns *et al.*¹⁴ Ascorbic acid formed from gulonolactone by this enzyme was identified by paper chromatography, chromatography on Dowex 1-formate, and recrystallization of the chromatographed ascorbic acid to constant specific activity.¹⁵ Rigorous identification of ascorbic acid is absolutely imperative in studies of biosynthesis, as is pointed out later.

When limiting amounts of substrate are used, the reaction proceeds according to the stoichiometry shown in Reaction 3.¹⁵ The reaction occurs with gulonolactone but not with free gulonate as substrate.

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Ascorbate is formed from free gulonate, however, if the microsomes are supplemented with a soluble heat-labile factor obtained from the soluble supernatant fraction of rat liver homogenates.¹⁶ No cofactor is required. On the other hand, this soluble factor inhibits the oxidation of gulonolactone to ascorbate by the microsomes. These findings suggested that the active factor in the soluble fraction is aldonolactonase, presumably capable of acting reversibly. Experimental support for such a role of the aldonolactonase has been provided by the finding that during the course of a 110-fold purification of the enzyme from the soluble fraction of rat liver, the ratio of the aldonolactonase activity to the activity in stimulating the formation of ascorbate from gulonate by the microsomes remained essentially constant. Further support of the ability of aldonolactonase to catalyze the lactonization of gulonate has been provided by the finding that the enzyme is able to catalyze the *net accumulation* of gulonolactone from gulonate.

In this experiment, alkaline hydroxylamine was added at the end of the incubation period, and corrections were made for the amount of color due to nonenzymatic lactonization, enzyme, and possible "trapping" by the enzyme after the addition of the hydroxylamine reagent. In this manner we have shown the net accumulation of a true equilibrium quantity of the lactone, amounting to some 28 per cent of the added gulonate at pH 4 and 5.¹⁷ The purified enzyme also catalyzes the formation of hydroxamic acids when a hydroxylamine trap is included in the incubation mixture in agreement with the work of Yamada¹⁸ and Chatterjee *et al.*⁵ However, the formation of a hydroxamic acid from gulonate under "trapping" conditions does not provide evidence that the lactonase reaction is necessarily reversible. Formation of a hydroxamate with a hydroxylamine "trap" indicates only the activation of a carboxyl group but not necessarily the ability to carry out net formation of a lactone or ester since Lipmann and Tuttle¹⁹ have demonstrated that esterases are capable of forming hydroxamates from simple organic acids under conditions in which no alcohol component is included in the enzyme incubation medium.

Gulonate has been identified as the hydrolysis product of gulonolactone by coupling the aldonolactonase reaction with either the TPN-L-gulonate dehydrogenase or the DPN-L-gulonate dehydrogenase reactions. When L-gulonolactone is substrate, a stimulation in the rate of both reactions is produced on addition of lactonase.¹⁶ The purified lactonase is capable of catalyzing the hydrolysis of several lactones in addition to gulonolactone and may therefore have additional roles in intermediary metabolism.

It has been postulated by Yamada *et al.*⁴ on the basis of a study of the distribution of aldonolactonase in animal tissues that a deficiency of aldonolactonase in animals that require dietary ascorbate might be the reason that these species are unable to synthesize ascorbate. This hypothesis is not supported by work in this laboratory. It was noted by Hassan and Lehninger²⁰ that addition of the soluble fraction of guinea pig liver to a rat liver microsome preparation could cause the conversion of gulonate into ascorbate. It is now established that the active factor in the soluble fraction is aldonolactonase, which Winkelman and Lehninger²¹ have found to be present in guinea pig and monkey livers although in somewhat smaller amounts than in other mammalian livers. The claim of Yamada *et al.*⁴ that primates do not possess an aldonolac-

onase and that guinea pigs possess a rather different kind of aldolactonase must, in our opinion, be qualified because of the rather low substrate concentrations used in their assay system. In addition, the fact that the total lactonase activity of tissue homogenates is of a much higher order of magnitude in molar terms than the gulonolactone oxidase of microsomes also weakens the argument that the inability of certain species to synthesize ascorbate is due to a deficiency of lactonase.

On the other hand, our studies support the hypothesis that the oxidase reaction is the site of the genetic deficiency in primates and guinea pigs, which are unable to synthesize their own ascorbate. Hassan and Lehninger²⁰ first provided evidence that the deficiency was in the liver microsomes, and later Burns *et al.*¹⁴ identified the deficient reaction as L-gulonolactone oxidase. Since experiments in which primate or guinea pig liver extracts were added to gulonolactone oxidase from rat liver failed to provide any evidence for an inhibitor of the oxidase, it would appear that the failure of guinea pig or primate liver to oxidize gulonolactone to ascorbate is due to a deficiency of the oxidase per se and not due to an inhibitor of this enzyme.¹¹

More critical evidence on the nature of the genetic defect has come from closer examination of the oxidase. It is possible to study the dehydrogenase component of the gulonolactone oxidase system¹⁵ using an aerobic optical assay with phenazine methosulfate as electron acceptor. Phenazine methosulfate is the electron acceptor for the dehydrogenase component, and oxygen is the electron acceptor for the complete oxidase system. From rat liver we have obtained active preparations of gulonolactone dehydrogenase that do not have gulonolactone oxidase activity. It therefore appears likely that the dehydrogenase can be separated from one or more electron carriers during the purification or, as an alternative, that the ability of the oxidase to react with oxygen is lost during purification without loss in reactivity with the dye. Using this assay, we have found that guinea pig and primate liver extracts and microsomes do not contain the dehydrogenase component of the oxidase system. However, it is not possible to conclude that the genetic deficiency is due solely to the lack of the dehydrogenase component of the oxidase system since it is possible that there is also a deficiency in one or more electron carriers.

In conclusion, we should like to refer to our earlier hypothesis that 3-ketogulonate is a common intermediate in the formation of both L-ascorbate and xylulose by the DPN-linked enzyme.¹ This suggestion was based on formation of a Roe chromogen during this reaction and a substance chromatographing on paper with the same R_f as L-ascorbic acid. We have since found that this substance is not identical with authentic L-ascorbic acid on careful chromatography of the C¹⁴-labeled material on Dowex 1.²² The original hypothesis was then abandoned. However, it now seems probable that the Roe chromogen formed in the DPN-L-gulonolactone dehydrogenase reaction is that of ketogulonate, which Ashwell *et al.* (elsewhere in this monograph) have demonstrated is accumulated in this reaction or in some transformation product of it.

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ENZYMATIC STUDIES ON THE METABOLISM OF URONIC AND ALDONIC ACIDS RELATED TO L-ASCORBIC ACID IN ANIMAL TISSUES*

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The biosynthesis of L-ascorbic acid from D-glucose in animal tissues has been studied by many workers, affording evidence that D-glucuronic acid, L-gulonic acid, or their lactones are converted to L-ascorbic acid.¹⁻⁵ On the other hand, it was proved that L-xylulose is formed from L-gulonic acid,⁶⁻⁸ and the glucuronic acid cycle was proposed.⁹ Thus it seems to be necessary to study the metabolism of these uronic and aldonic acids enzymatically to clarify the possible pathway as a multienzyme system of reactions.

The degradation of L-ascorbic acid in animal tissues has been studied for many years. It was proved that L-ascorbic acid can be metabolized back to D-glucose^{10,11} and that the degradation of L-ascorbic acid takes place to form L-lyxonic acid¹² or L-xylulose.¹⁰ These findings suggest the importance of studying the degradation of 2,3-diketo-L-gulonic acid formed by the oxidation of L-ascorbic acid in animal tissues.

Thus the chief object of this paper is to study the enzymatic reactions concerning the metabolism of D-glucuronic and L-gulonic acids and their lactones, as well as of 2,3-diketo-L-gulonic acid. The enzymes studied are: triphosphopyridine nucleotide (TPN) L-hexonate dehydrogenase, which oxidizes L-gulonic acid to D-glucuronic acid; diphosphopyridine nucleotide (DPN) L-gulonate dehydrogenase, which oxidizes L-gulonic acid to L-xylulose with carbon dioxide evolution; lactonase I and II, which hydrolyze the lactone ring of D-glucuronolactone and L-gulonolactone; L-gulonolactone oxidizing enzyme, which converts L-gulonolactone to L-ascorbic acid; and 2,3-diketoaldonate decarboxylase, which decomposes 2,3-diketo-L-gulonic acid to L-lyxonic and L-xylonic acids with evolution of carbon dioxide (FIGURE 1). These enzymes are all present in the soluble fraction of liver homogenate with the exception of lactonase II and L-gulonolactone oxidizing enzyme, which are present in the microsomes.

Materials and Methods

TPN L-hexonate dehydrogenase. This enzyme was partially purified from rat liver as follows.^{13,14} The supernatant fraction from rat liver homogenate was subjected to ammonium sulfate fractionation (0.40 to 0.50 saturation), dialysis, and fractionation on a diethylaminoethyl-(DEAE)-cellulose column. The second fraction, eluted with 0.02 M tris(hydroxymethylamino)methane buffer (Tris buffer), pH 7.5, usually showed a specific activity 350 to 500 times that of the original extract. The enzyme activity was estimated by the change in optical density at 340 m μ after incubation in air for 10 min. at 37° C. of a re-

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action mixture consisting of 10 μ moles of substrate, 0.5 μ moles of TPN or TPNH, 50 μ moles of phosphate buffer, pH 7.0, and the enzyme preparation, in a total volume of 3.0 ml. The unit of enzyme was defined as the change of this optical density $\times 10^3$.

DPN L-gulonate dehydrogenase. This enzyme was partially purified from guinea pig liver homogenate.¹⁵ The supernatant fraction was subjected to: ammonium sulfate fractionation (38.5 to 53.5 per cent v/v); dialysis of the enzyme

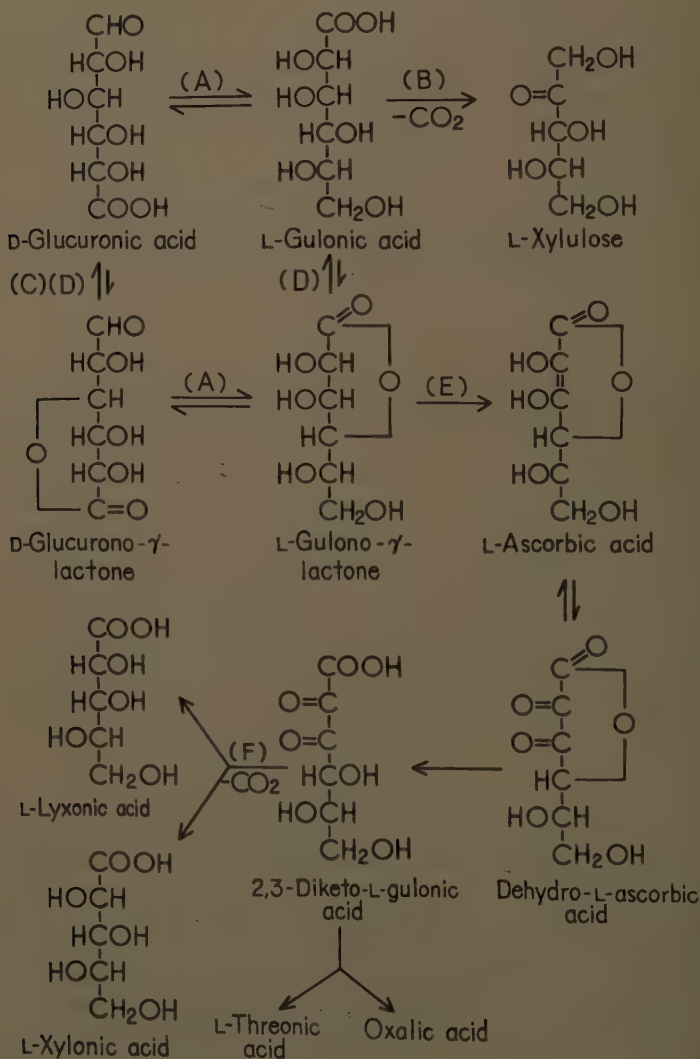


FIGURE 1. Enzymatic reactions of uronic and aldonic acids related to L-ascorbic acid. Key: (A) TPN L-hexonate dehydrogenase; (B) DPN L-gulonate dehydrogenase; (C) lactonase II (microsomal); (D) lactonase I (soluble); (E) L-gulonolactone oxidizing enzyme (microsomal); (F) 2,3-diketoaldonate decarboxylase.

solved in 0.04 *M* Tris buffer (*pH* 7.5) containing 1×10^{-3} *M* ethylenediaminetetraacetate (EDTA); negative adsorption with DEAE-cellulose; and a second ammonium sulfate fractionation (45 to 60 per cent v/v). The enzyme was purified by this procedure to about 20 times that of the original extract. To separate this enzyme from TPN L-hexonate dehydrogenase, sorbitol dehydrogenase, and lactic dehydrogenase, further purification was performed by column chromatography using DEAE-cellulose with Tris buffer as a developing solvent. The enzyme activity was estimated by the change in optical density at 340 $m\mu$ after incubation in air for 5 min. at 25° C. of a mixture of 10 μ moles substrate, 0.65 μ mole of DPN, 0.2 μ mole of $MnCl_2$, 50 μ moles of Tris buffer, *pH* 8.4, and the enzyme preparation in a total volume of 3.0 ml. The unit of enzyme was defined as the change of this optical density $\times 10^3$.

Lactonase I and II. Lactonase I (soluble) was partially purified¹⁶ from the acetone-dried powder of bovine liver by extraction with cold water, heat treatment (63° C.), ammonium sulfate fractionation (0.52 to 0.67 saturation), dialysis, and fractionation with acetone (40 to 55 per cent v/v) at *pH* 6.8, and chromatography on DEAE-cellulose column, eluted with 0.01 *M* Tris buffer at *pH* 7.35 containing 0.02 *M* NaCl. The specific activity of the preparation thus purified was about 20 times that of the water extract of the acetone-dried powder. Lactonase II (microsomal) was investigated using the microsomal fraction prepared by the method of Hogeboom.¹⁷ The activity of lactone hydrolysis was observed manometrically in an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide by the carbon dioxide evolution from a mixture of 30 μ *M* of the substrate, 10 μ moles of $MgSO_4$, 0.3 μ mole of glutathione, 40 μ moles of bicarbonate, and 0.2 ml. of the enzyme solution in a total volume of 3.0 ml. at *pH* 7.2.

The unit of enzyme activity was defined as the activity that produced 1 μ mole of carbon dioxide under the above conditions during 10 min. To observe the reverse reaction, that is, the formation of lactones,¹⁸ the reaction mixture consisted of 200 μ moles of the substrate, 120 μ moles of hydroxylamine, 20 μ moles of $MgCl_2$, 0.1 μ mole of glutathione, 0.5 ml. of the enzyme preparation, and 500 μ moles of Tris maleate buffer, *pH* 6.1, in a total volume of 6.0 ml. After incubation at 37° C. for 60 min. in air, the hydroxamate formed from the lactone was measured colorimetrically (in the supernate) at 540 $m\mu$ after the addition of a reagent containing HCl, trichloroacetic acid, and $FeCl_3$.

Microsomal L-gulonolactone oxidizing enzyme. The activity of this enzyme was observed in microsomes prepared from rat liver.¹⁷ The assay conditions were as follows:¹⁹ The incubation was carried out in Warburg flasks containing 10 μ moles of substrate, microsomes from 0.4 gm. of rat liver, and 150 μ moles of phosphate buffer, *pH* 7.0, in a final volume of 3.0 ml., in an atmosphere of oxygen for 30 min. at 37° C. The oxygen consumption was estimated, and the lactic acid formed was determined by the method of Roe and Kuether.²⁰

*3-Diketoaldonate decarboxylase.*²¹ This enzyme was purified from the supernatant fraction of rat liver homogenate. It was subjected to ammonium sulfate fractionation (0.55 to 0.70 saturation), dialysis, centrifugation, and fractionation on a DEAE-cellulose column. The fraction eluted with 0.05 *M* sodium chloride in 0.005 *M* phosphate buffer, *pH* 7.5, usually showed a specific activity 100 times that of the supernate. The activity of this enzyme was determined manometrically using 250 μ moles of phosphate buffer, *pH* 6.8, with

50 μ moles of substrate in a total volume of 2.5 ml. incubated for 30 min. at 37° C. in an atmosphere of nitrogen. It was also estimated by determining the quantity of remaining diketogulonate with 2,4-dinitrophenylhydrazine.²⁰

TABLE 1
SUBSTRATE SPECIFICITY OF TPN L-HEXONATE DEHYDROGENASE
Reduction Activity

Substrate	Activity unit	Substrate	Activity unit
D-Glucuronate	355	D-Glucosone	405
D-Glucurono- γ -lactone	420	D-Galactosone	488
Ethyl D-glucuronate	327	D-Xylosone	11
D-Glucuronamide	138	L-Arabinosone	26
D-Galacturonate	449	Methylglyoxal	974
Methyl D-galacturonate	370	D-Fructuronate	24
D-Mannuronate	414	D-Tagaturonate	15
D-Mannurono- γ -lactone	445	2-Keto-L-gulonate	18
L-Iduronate	524	Methyl 2-keto-L-gulonate	27
L-Idurono- γ -lactone	580	2-Keto-D-gluconate	8
D-Xyliuronate	0	5-Keto-D-gluconate	26
D-Lyxuronate	12	Keto-DL-erythronate	15
L-Arabiuronate	17	D-Glucose	0
L-Threuronate	280	D-Galactose	0
Methyl diacetyl L-threuronate	728	D-Mannose	0
DL-Tartronic semialdehyde	332	L-Galactose	0
Glyoxylate	556	D-Ribose	54
Glutaric semialdehyde	380	D-Arabinose	0
Succinic semialdehyde	625	D-Xylose	0
Malonic semialdehyde	460	D-Lyxose	0
Methylformyl acetate	510	L-Arabinose	0
Glutaric dialdehyde	585	L-Xylose	0
Succinic dialdehyde	520	L-Lyxose	0
Malonic dialdehyde	625	D-Erythrose	412
Glyoxal	651	D-Threose	172
n-Valeraldehyde	433	D-Glyceraldehyde	484
n-Butyraldehyde	123	L-Glyceraldehyde	592
Propionaldehyde	49	DL-Glyceraldehyde	903
Acetaldehyde	16	Glycol aldehyde	119
Crotonaldehyde	61	L-Rhamnose	0
Benzaldehyde	446		
Aldol	118	L-Fucose	0
Acrolein	37	D-Xylodialdopentofuranose	446
DL-lactic aldehyde	465	2,3,4,5,6-Pentaacetyl D-glucose	340
Hydroacryl aldehyde	620	2,3,4,6-Tetraacetyl D-glucose	8
		D-Glucose-6-phosphate	80
		D-Galactose-6-phosphate	47
		DL-Glyceraldehyde-3-phosphate	115

Substrate Specificity of the Enzymes

The substrate specificity of the above enzymes was studied, and the results are summarized in TABLES 1 and 2.

*TPN L-hexonate dehydrogenase.** TPN-specific L-hexonate dehydrogenase was reported by Ul Hassan and Lehninger (1956)⁴ and Ishikawa and Noguchi (1957)⁷ as the enzyme that oxidizes L-gulonic acid to form D-glucuronic acid. Mano and his co-workers (1959)¹⁴ reported that this enzyme acts to form L

* Consult TABLES 1 and 2.

gulonolactone from D-glucuronolactone in the presence of TPNH without spontaneous or enzymatic formation of D-glucuronic acid as an intermediate.

As shown in TABLE 1, in the reduction process this enzyme acts on alduronic acids of which the carbon number varies from six to three with the exception of

TABLE 2
SUBSTRATE SPECIFICITY OF THE ENZYMES

Enzyme.....	TPN-L-hexonate dehydrogenase*		Lactonase I†	DPN-L-gulonate dehydrogenase‡		L-Gulonolactone oxidizing enzyme	
Reaction.....	Oxidation		Hydrolysis of lactone	Oxidation		Formation of L-ascorbic acid	
Activity unit.....	L-Gulonate = 100		L-Gulonolactone = 100	L-Gulonate = 100		L-Gulonolactone = 100	
Substrate	Acid	γ -Lactone	γ -Lactone	Acid	γ -Lactone	Acid	γ -Lactone
α -Glucuheptonic	0	0	46	0	0	0	0
β -Glucuheptonic	0	0	—	0	0	0	0
Allonic	0	0	71	0	0	0	0
Altronic	0	0	42	0	0	0	17
Gluconic	0	0 (8, 0)	190 (8, 29)	19.0	0	0	0
Mannonic	18.7	11	31	5.1	0	0	35
Gulonic	0	0	278	0	0	0	0
Idonic	0	0	45	0	0	0	22
Galactonic	0	0	160	0	0	0	0
Talonic	0	0	25	0	0	0	20
Gluconic	41	— (8, 35.4)	20	0	0	0	16
Mannonic	69.5	30	16	0	0	0	0
Gulonic	100	46.5	100	100	0	0	100
Idonic	109	62.5	10	68.8	0	0	0
Galactonic	49	24	139	0	0	0	67
Rhammonic	0	0	3	—	0	0	0
Ribonic	0	0	11	0	0	0	0
Arabonic	0	0	(+)	0	0	0	27
Xylonic	0	0	70	15.5	0	0	0
Lyxonic	0	0	190	44.6	0	0	46
Arabonic	0	0	115	0	0	0	0
Xylonic	0	0	21	0	0	0	10
Lyxonic	0	0	60	0	0	0	0
Erythronic	0	0	(+)	17.1	—	0	—
Threonic	0	0	(+)	7.8	0	0	0
Glyceric	0	0	—	0	—	0	—

* Other substrates tested for TPN L-hexonate dehydrogenase: glycolate, *N*-acetylneurinate, β -hydroxypropionate—0; D-sorbitol, D-mannitol, mesodulcitol, mesoadonitol, L-zeitol, glycerol, ethylene glycol, ethanol—0.

† Other substrates tested for lactonase I; D-glucuronolactone—31; D-mannuronolactone—L-iduronolactone—66; ethyl D-glucuronate, triacetin, glycerophosphate, acetyl choline—0. Key: (+) means that the activity was observed but the exact data could not be obtained the hydrolysis also occurred nonenzymatically.

‡ Other substrates tested for DPN L-gulonate dehydrogenase: 2-keto-D-gluconate, 5-keto-gulonate, 2-keto-L-gulonate, methyl 2-keto-D-gulonate, D-glucuronate—0; DL- β -hydroxy-urate—14.4.

e; D-glucuronate, D-galacturonate, D-mannuronate, L-iduronate, L-threuronate, and DL-tartronic semialdehyde. It acts neither on the corresponding uronates nor on other keto acids. It also catalyzes the reduction of such derivatives as lactones, esters, and amides of the above-mentioned alduronic

acids, and the reduction of glyoxylate, malonic, succinic, and glutaric semi-aldehydes, and of various osones derived from hexose. Methylglyoxal was proved to be the most active among the compounds tested. Of the various aldomonosaccharides examined, those with carbon numbers of three and four were active, while those with carbon numbers of five and six were not, with the exception of a slight activity of D-ribose. Glycol aldehyde was also active. Various ketomonosaccharides did not serve as substrate. The enzyme had considerable activity on dialdehydes such as glyoxal and malonic, succinic, and glutaric dialdehydes. Typical aldehydes such as acet-, propion-, *n*-butyr-, and *n*-valeraldehyde were somewhat reactive; the activities being almost in parallel with the chain length. These observations suggest that this enzyme attacks compounds having a free aldehyde group, with the activity for uronate to be considered possibly as a special case. This broad substrate specificity is very close to that of "aldehyde reductase" reported by Hers (1960),²² except for the ineffectiveness of ketomonosaccharides. The reaction products were identified by paper chromatography with four solvent systems as follows: L-gulonate from D-glucuronate; L-gulonolactone from each D-glucurono- γ -lactone and ethyl D-glucuronate; L-galactono- γ -lactone from methyl D-galacturonate; and D-xylose from D-xylodialdopentofuranose. The formation of L-gulono- γ -lactone from D-glucurono- γ -lactone and ethyl D-glucuronate was also confirmed by the coupling reaction with L-gulonolactone oxidizing enzyme leading to the production of L-ascorbate. The participation of lactonase and esterase in this reaction was excluded. The possibility that D-fructose is formed from D-glucosone and acetol from methylglyoxal was suggested. The formation of 5-ketoaldonate as an intermediate in the conversion of uronate to aldonate, which was once suggested,^{23,24} seemed not to take place, because this substance was not active as a substrate in the process of reduction with this enzyme.

As shown in TABLE 2, in the dehydrogenation process the enzyme was proved to act exclusively on the L-form of both hexonic acids and their lactones, namely, L-gluconate and its δ -lactone, L-galactonate, L-mannonate, L-gulonate, and L-idonate, and their γ -lactones, with the exception of minute activity of D-mannonate and its γ -lactone. L-Rhamnonate and its γ -lactone were ineffective as substrates. These findings suggest that for the activity of this enzyme on L-hexonate the levo configuration of the hydroxyl group at C-5 and the presence of an alcohol group at C-6 are essential. Because of such a strict substrate specificity in the dehydrogenation reaction we propose the term TPN L-hexonate dehydrogenase for this enzyme.²⁵ The reaction product formed from L-galactonate was D-galacturonate, and the formation of L-xylulose was not observed, as was once reported to be produced from L-galactonate by TPN enzyme.²⁶

*DPN L-gulonate dehydrogenase.** DPN-specific L-gulonate dehydrogenase was reported by Ishikawa and Noguchi (1957),⁷ and Bublitz *et al.* (1958)⁸ and had been partially purified by Ishikawa (1959)¹⁵ and Ashwell *et al.* (1959).²⁷ It acts on L-gulonate to produce L-xylulose.

The substrate specificity was examined chiefly with aldonic acids and some related compounds.²⁸ Of these compounds tested, L-gulonate, D-gluconate, D-mannonate, L-idonate, D-xylonate, D-lyxonate, DL-erythronate, L-threonate, and

* Consult TABLE 2.

DL- β -hydroxybutyrate were effective as substrates. In all these effective compounds the hydroxyl group of the C-3 has a levo configuration, and the hydrogen of the C-4 occupies the same side as the hydroxyl group of the C-3. 2-Keto as well as 5-keto aldonic acids and those compounds in which the terminal carbon was an aldehyde did not serve as a substrate. Smiley and Ashwell (1960)²⁹ reported recently that this enzyme is active on all hexonic, pentonic, and tetric acids examined in which the hydroxyl group of the C-3 has a levo configuration, and the reverse reaction, from 2,3-diketo-L-gulonate, 2,3-diketo-D-gluconate, 2,3-diketo-D-glucose, and acetoacetate to the corresponding C-3 hydroxy acids, takes place. In our study, however, the enzyme was not active upon such compounds as D-galactonate, D-talonate, and L-arabonate, in which the hydroxyl group of the C-3 has a levo configuration. Therefore, the simple condition of a C-3 levo-hydroxyl group might not be sufficient, but the levo-hydroxyl at C-3 and hydrogen on the same side at C-4 might be necessary for the substrate. It was not possible to observe the reverse reaction that Smiley and Ashwell²⁹ reported, in so far as was attempted under several conditions. This difference might be attributed to the difference of the source of the enzyme.

By the action of DPN L-gulonate dehydrogenase on D-gluconate, D-ribulose is produced. Using uniformly labeled D-gluconate-C¹⁴ as a substrate, the product was proved to be D-ribulose by separation with Dowex 1 borate column chromatography and by crystallization as *o*-nitrophenylhydrazone with constant specific activity.³⁰ This reaction throws light upon one of the pathways of the formation of D-ribulose from D-glucose without phosphorylation.

Lactonase I and II.* Presence of glucuronolactonase activity was first demonstrated by Eisenberg and Field (1956)³¹ in rat liver homogenate and slices. Further, Ul Hassan and Lehninger (1957)⁴ observed the presence of gulonolactonase activity in addition to the glucuronolactonase activity in rat liver extract. Winkelman and Lehninger (1958)³² and Yamada *et al.* (1959)³³ demonstrated that there exist soluble lactonase (aldonolactonase, according to Winkelman and Lehninger;³² lactonase I, according to Yamada *et al.*) and microsomal lactonase (uronolactonase, according to Winkelman and Lehninger;³² lactonase II, according to Yamada *et al.*³³). Yamada¹⁶ and Yamada *et al.*³³ reported that microsomal lactonase acts on D-glucuronolactone but not on D-gulonolactone, L-gulonolactone, D-mannonolactone, D-mannuronolactone, or L-gluconolactone, whereas the soluble lactonase has a broad substrate specificity and hydrolyzes L-gulonolactone about three times as much as D-glucuronolactone in most cases. L-galactonolactone, D-galactonolactone, L-gluconolactone, D-gluconolactone, D-mannonolactone, D-mannuronolactone, D-glucuronolactone, and D-gluconolactone were all active as substrates of soluble lactonase. The enzyme showed no esterase activity tested with triacetin, glycerophosphate, acetylcholine, and ethyl D-glucuronate as substrates. Yamada (1959)¹⁸ also reported that the reverse reaction, that is, the formation of lactones from L-gulonic acid and D-glucuronic acid, was also observed to take place when a trapping agent such as hydroxylamine was added to the reaction system. The lactonization of L-galactonic acid also took place under the same conditions. The enzyme was found to act more effectively on L-gulonic and L-galactonic acids than on D-glucuronic acid.

* Consult TABLE 2.

*L-Gulonolactone oxidizing enzyme (microsomal).** Burns *et al.* (1956),³⁴ Chang and Tung (1957),³⁵ Kanfer *et al.* (1959),³⁶ Bublitz and Lehninger (1959),³⁷ and Chatterjee *et al.* (1960)³⁸ reported that the enzyme capable of catalyzing the conversion of *L*-gulonolactone to *L*-ascorbic acid is present in rat liver microsomes. Chatterjee *et al.*³⁸ termed this enzyme *L*-gulono-oxidase, and Trucco *et al.* (1960)³⁹ termed it *L*-gulonolactone oxidase. Kanfer *et al.*³⁶ demonstrated that the microsomal enzyme possessed a configurational specificity for the levo configuration of the C-2 hydroxyl group.

The enzyme oxidizes *L*-gulonolactone to *L*-ascorbic acid in the presence of oxygen. Anaerobically the reaction takes place in the presence of a dye whose

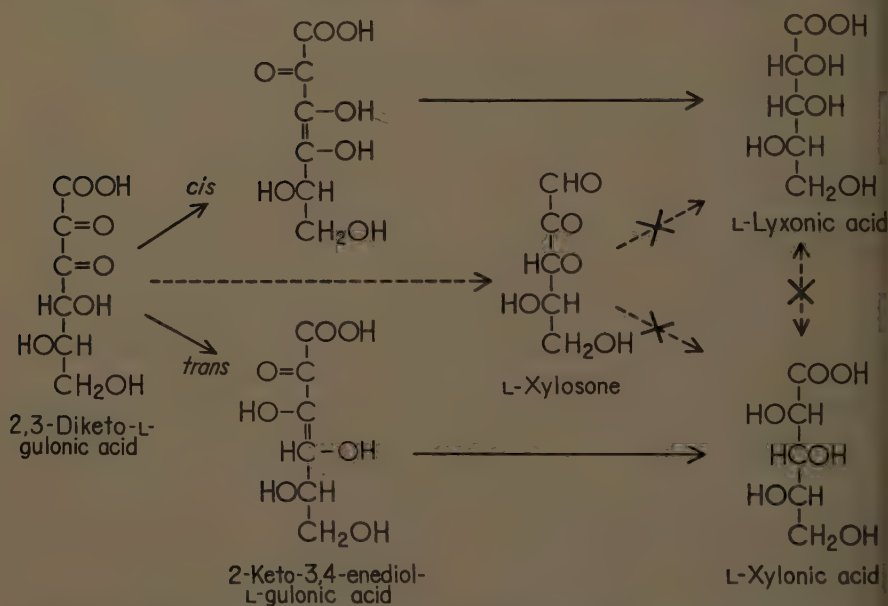


FIGURE 2. Presumed reaction mechanism of 2,3-diketoaldonate decarboxylase. Symbols: —————> possible route; - - - - -> doubtful route; - - - X - - -> neglected route.

redoxpotential is over 200 mv such as 2,6-dichlorophenolindophenol. The enzyme appears to be specific for the levo C-2 hydroxyl group of pentonic and hexonic acid lactones.¹⁹ The free acids corresponding to the tested lactones and *L*-rhamnolactone were completely inactive. The lactone structure and the terminal alcohol group seem to be essential for the activity. In general, lactones that have the lactone ring of dextroposition are more active than those with levoposition. The activity for *L*-xylonolactone suggests that the enzyme might take part in the catabolism of *L*-ascorbic acid in animal tissues, because *L*-xylosonic acid is produced by the decarboxylation of 2,3-diketo-*L*-gulonic acid as described below.

2,3-Diketoaldonate decarboxylase.† Chan *et al.* (1958),¹⁰ Burns *et al.* (1958),⁴⁰

* Consult TABLE 2.

† See FIGURE 2.

and Kanfer *et al.* (1959)¹² published the findings that enzymes that occur in the liver and kidney of guinea pig and rat decarboxylate dehydroascorbic acid; Chan *et al.*¹⁰ and Kanfer *et al.*¹² reported the formation of L-xylose and L-lyxonic acid respectively. The enzyme 2,3-diketoaldonate decarboxylase was purified from rat liver by Kagawa *et al.*²¹ in our laboratory. It was found in the kidney and liver of rat, dog, and hog. It catalyzed the decarboxylation of 2,3-diketo-gulonic acid. Among other keto acids tested, substrates were limited to 2,3-diketoaldonic acids derived from ascorbic acid homologues such as L- and D-ascorbic acids, araboascorbic acids, erythroascorbic acids, and D-glucoascorbic acid. Dehydro-L-ascorbic acid was also decarboxylated with a lag probably owing to its hydrolysis into 2,3-diketo-L-gulonic acid. The reaction products of 2,3-diketo-L-gulonic acid were put on gradient elution chromatography with Dowex 1 and identified as lyxonic acid and xylonic acid on a paper chromatogram with four solvent systems, using a pH indicator, periodate, and AgNO₃, or hydroxamate formation after their conversion into lactones. They were identified further by the benzimidazole derivatives obtained in crystalline form by the reaction with *o*-phenylenediamine. The nearly equimolecular formation of the two acids was observed by means of periodate oxidation and subsequent chromotrophic acid color reaction. Both of the acids were levorotatory. The fact that these two acids were obtained from this enzyme reaction suggests that the enolization of 2,3-diketo-L-gulonic acid takes place to form *cis*- and *trans*-2-keto-3,4-enediol-L-gulonic acids, which serve as true substrates of the decarboxylation, as the enzyme preparation had no epimerase activity and glucosone showed no activity as substrate (FIGURE 2).

Reconstructed System of the Enzymes

The formation of L-xylulose from D-glucuronic acid. When D-glucuronic acid was incubated with TPN L-hexonate dehydrogenase and DPN L-gulonate dehydrogenase, the formation of L-xylulose was observed. It was identified by the reaction of resorcinol and orcinol after treatment of Norit A and Dowex 1. Although the optimal pH of the DPN enzyme was over 8.0,¹⁵ it was observed that the coupling reaction with the TPN enzyme to form L-xylulose could take place at pH 7.5.

*The formation of L-ascorbic acid from L-gulonic acid.** It was observed that the rat liver slice can synthesize L-ascorbic acid from L-gulonolactone or L-gulonic acid. The reaction mixture consisted of L-gulonolactone or L-gulonic acid, MgCl₂, glutathione, and a liver slice in phosphate buffer (pH 6.8) incubated in air at 37° C. for 60 min. However, the guinea pig liver slice could not synthesize L-ascorbic acid under the same conditions (TABLE 3).

The microsomes of the liver of rats and rabbits and the kidneys of frogs, snakes, and pigeons could synthesize L-ascorbic acid from L-gulonolactone, but those of guinea pigs and men could not. The amount of formation of L-ascorbic acid by the microsomes from rat liver was decreased only to a small extent when soluble lactonase was added to the system. When L-gulonic acid was used as a substrate, microsomes alone could not synthesize L-ascorbic acid, while further addition of soluble lactonase permitted the L-ascorbic acid

* Consult TABLES 3 and 4.

formation (TABLE 4). Similar results were reported by Bublitz and Lehninger (1959)³⁷ and Chatterjee *et al.* (1960).³⁸ These findings suggest that the formation of a lactone ring by lactonase takes place, with microsomal L-gulonon-

TABLE 3
SYNTHESIS OF L-ASCORBIC ACID BY LIVER SLICE OF RAT AND GUINEA PIG

Liver slice	Reaction system*	Substrate	L-Ascorbic acid† found (μM /gm.)
Rat	Complete	—	0.02
Rat	Complete	L-Gulonolactone (10 μM)	4.2
Rat	Mg ⁺⁺ (-)	L-Gulonolactone (10 μM)	5.2
Rat	Complete	L-Gulonic acid (60 μM)	4.8
Rat	Mg ⁺⁺ (-)	L-Gulonic acid (60 μM)	1.8
Rat	Glutathione (-)	L-Gulonic acid (60 μM)	3.4
Guinea pig	Complete	L-Gulonolactone (10 μM)	0
Guinea pig	Complete	L-Gulonic acid (60 μM)	0

* The complete reaction system consisted of phosphate buffer, 400 μM , pH 6.8; MgCl₂, 10 μM ; glutathione, 1 μM ; substrate added as above; and slice, 330 mg.; in a total volume of 4.0 ml. was incubated for 60 min. at 37° C. in oxygen.

† When 1 μM of ascorbic acid was added to the enzyme system, 0.95 μM of it was found after 1-hour incubation.

TABLE 4
FORMATION OF L-ASCORBIC ACID FROM L-GULONATE AND L-GULONOLACTONE
BY RECONSTRUCTED ENZYME SYSTEM

Composition of enzyme system	Substrate	Ascorbic acid formed (μM)
Microsomes	L-Gulonolactone (10 μM)	3.6
Microsomes; lactonase I	L-Gulonolactone (10 μM)	2.6
Microsomes	L-Gulonate (60 μM)	0
Microsomes; lactonase I	L-Gulonate (60 μM)	1.8
Microsomes; lactonase I; TPN L-hexonate dehydrogenase	L-Gulonolactone (10 μM)	2.5
Microsomes; lactonase I; TPN L-hexonate dehydrogenase; DPN L-gulonate dehydrogenase	L-Gulonolactone (10 μM) L-Gulonate (60 μM)	2.5 1.8

The reaction system consisted of microsomes from 600 mg. of wet liver; gulonolactonase, 40 units; TPN L-hexonate dehydrogenase, 1300 units; DPN L-gulonate dehydrogenase, 70 units; phosphate buffer, 300 μM , pH 6.8; DPN, 1 μM ; TPN, 1 μM ; MgCl₂, 10 μM ; and glutathione, 1 μM ; in a total volume of 4.5 ml. was incubated in O₂ at 37° C. for 60 min.

lactone oxidizing enzyme acting as a trapper, and that lactonization by means of lactonase is an essential step in the formation of L-ascorbic acid from L-gulonate. The concurrent presence of TPN L-hexonate dehydrogenase or DPN L-gulonate dehydrogenase did not inhibit the formation of L-ascorbic acid from L-gulonic acid.

*The formation of L-ascorbic acid from D-glucuronolactone.** The formation of L-gulonolactone was observed stoichiometrically when D-glucuronolactone was incubated with TPN L-hexonate dehydrogenase, without the intermediate formation of free glucuronic acid.¹³ When microsomes from rat liver were added to this reaction mixture, the formation of L-ascorbic acid was observed (TABLE 5). The reaction was inhibited to some extent by the addition of barbiturate, which is a specific inhibitor of TPN L-hexonate dehydrogenase.¹⁴ If D-glucuronic acid was used as a substrate instead of D-glucuronolactone, the formation of L-ascorbic acid was not observed. These findings suggest that lactonization does not take place under these conditions.

Chatterjee *et al.* (1957, 1958)^{41,42} reported that L-ascorbic acid was formed from L-glucuronolactone by a goat liver preparation in the presence of KCN.

TABLE 5
FORMATION OF L-ASCORBIC ACID FROM D-GLUCURONOLACTONE AND D-GLUCURONATE
BY RECONSTRUCTED ENZYME SYSTEM

Composition of enzyme system	Substrate (40 μM)	L-Ascorbic acid formed (μM)
TPN L-hexonate dehydrogenase	D-Glucuronate	0
TPN L-hexonate dehydrogenase; microsomes	D-Glucuronate	0
	D-Glucuronolactone	0.9
TPN L-hexonate dehydrogenase; microsomes; + barbital (20 μM)	D-Glucuronolactone	0.5
TPN L-hexonate dehydrogenase; microsomes; lactonase I	D-Glucuronate	1.2
TPN L-hexonate dehydrogenase; microsomes; lactonase I; + lycorine (20 μM)	D-Glucuronate	0.8

The reaction system consisted of glycylglycine buffer, 100 μM , pH 7.3; TPN 6 μM ; $MnCl_2$ 4 μM ; nicotinamide, 250 μM ; D, L-isocitrate, 50 μM ; isocitric dehydrogenase, 25 units and the enzyme as specified in a total volume of 4.5 ml. was incubated for 60 min. at 37° C. in O_2 .

Recently Suzuki *et al.* (1960)⁴³ demonstrated that L-gulonolactone is formed as an intermediate in this case also, and L-ascorbic acid is formed by the action of L-gulonolactone oxidizing enzyme in the microsomes.

*The formation of L-ascorbic acid from D-glucuronic acid.** For the formation of L-ascorbic acid from D-glucuronic acid, the presence of lactonase was found to be necessary in addition to TPN L-hexonate dehydrogenase and L-gulonolactone oxidizing enzyme (TABLE 5). As stated above, lactonase I was observed to act on L-gulonic acid more effectively than on D-glucuronic acid.¹⁸ Thus it might be concluded that L-gulonic acid is first formed from D-glucuronic acid and then the lactonization of L-gulonic acid takes place, after which L-gulonolactone oxidizing enzyme acts to produce L-ascorbic acid. The same reconstructed system of enzymes could also act on D-galacturonic acid to form L-ascorbic acid. The amount of formation of L-ascorbic acid in this case was

* Consult TABLE 5.

almost the same as in the case of D-glucuronic acid. From similar findings in the case of D-glucuronic acid, it might be thought that L-galactonic acid and L-galactonolactone would be formed as the intermediates in this case, although Mapson (1960)⁴⁴ suggested the formation of some galacturonic and galactonic acid derivatives in plant tissues. Lycorine, an alkaloid from *Lycoris radiata*, was reported to inhibit the biosynthesis of L-ascorbic acid *in vivo* in rats.⁴⁵ We found that lycorine inhibits the soluble lactonase; this might well be the mechanism of the decrease in the biosynthesis of L-ascorbic acid.

The formation of L-ascorbic acid homologues from L-ascorbic acid. When L-ascorbic acid is oxidized in animal tissues, 2,3-diketo-L-gulonic acid is formed. The action of 2,3-diketo-aldonate decarboxylase on this compound produces L-lyxonic and L-xylonic acids.²¹ When L-lyxonic and L-xylonic acids are lactonized either by soluble lactonase or nonenzymatically, L-lyxonolactone and L-xylonolactone should be formed, and L-xylonolactone would produce L-erythroascorbic acid by the action of L-gulonolactone oxidizing enzyme. It was observed that a substance similar to L-ascorbic acid was produced from L-xylonic acid in the presence of soluble lactonase and L-gulonolactone oxidizing enzyme. If 2,3-diketoaldonate decarboxylase acted on this substance, L-erythronic acid and L-threonic acid would be produced, from which dihydroxyacetone would be formed by the action of DPN L-gulonate dehydrogenase. The reaction might participate in the pathway by which L-ascorbic acid is metabolized back to D-glucose, although it might serve only as a salvage route, considering the predominant incorporation of C¹⁴ of L-ascorbate-6-C¹⁴ into C-1 and C-6 of glucose reported to occur in guinea pig liver.^{10,11}

Summary

(1) The following enzymes participating in the metabolism of the uronic and aldonic acids related to L-ascorbic acid were studied: TPN L-hexonate dehydrogenase; DPN L-gulonate dehydrogenase; lactonase I (soluble) and II (microsomal); L-gulonolactone oxidizing enzyme (microsomal); and 2,3-diketoaldonate decarboxylase.

(2) The substrate specificity of each enzyme was studied. TPN L-hexonate dehydrogenase has a broad specificity in the reduction activity, but it is specific for L-hexonate in its oxidation activity. It acts on D-glucuronolactone to produce L-gulonolactone. DPN L-gulonate dehydrogenase acts upon hexonic, pentonic, and tetronic acids with a levo-hydroxyl at C-3 and a hydrogen on the same side at C-4. For instance it acts on L-gulonate and D-gluconate to produce L-xylulose and D-ribulose, respectively. Lactonase II, which occurs in the microsomes, is active on D-glucuronate but not active on L-gulonate. Typical lactonase I that occurs in the supernate acts on both in the ratio of 1:3. It has a reverse action when a trapping system is present. L-Gulonolactone oxidizing enzyme is located in microsomes and is specific for the levo C-2 hydroxyl group of pentonic and hexonic acid lactone. 2,3-Diketoaldonate decarboxylase acts to decarboxylate the 2,3-diketoaldonic acids derived from ascorbic acid homologues.

(3) The reconstructed systems of the above enzymes were tested for the formation of L-xylulose and L-ascorbic acid. The coupling of TPN L-hexonate dehydrogenase and DPN L-gulonate dehydrogenase makes possible the for-

mation of L-xylulose from D-glucuronic acid. The coupling of lactonase I and L-gulonolactone oxidizing enzyme produces L-ascorbic acid from L-gulononic acid. When TPN L-hexonate dehydrogenase is added to this system the synthesis of L-ascorbic acid from D-glucuronic acid becomes possible. The action of 2,3-diketoaldonate decarboxylase that couples with lactonase, L-gulonolactone oxidizing enzyme and DPN L-gulonate dehydrogenase participates in the decomposition of L-ascorbic acid or the metabolism of L-ascorbic acid back to D-glucose.

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Addendum

After this manuscript was written, the paper of Kanfer *et al.* (1960)⁴⁶ appeared, which also proved the formation of L-lyxonic and L-xylonic acids from L-ascorbic acid in animal tissue.

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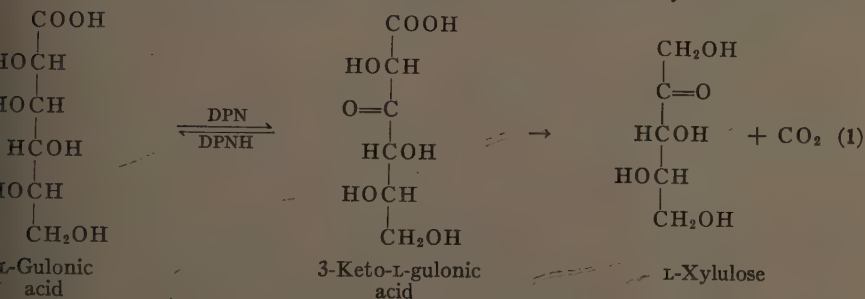
METABOLISM OF ASCORBIC ACID AND RELATED URONIC ACIDS, ALDONIC ACIDS, AND PENTOSES

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The outlines of the biosynthetic pathway involved in the formation of L-ascorbic acid in mammalian tissues are by now abundantly clear: as is evident from the overlapping reports describing it in such detail in this monograph. However, there are at least two closely related areas on which little or no information is currently available: (1) the controversial role of 3-keto-L-gulonic acid, and (2) the subsequent metabolic fate of L-ascorbic acid in animal tissues. Consequently this report will confine itself largely to a description of recent work in these areas. In addition, there has emerged from these studies a newer appreciation of the unusually broad but nevertheless uniquely restricted specificity requirements for each of the purified enzymes participating in these reactions. On the basis of these observations, simple rules can now be adduced whereby the reactivity of a given compound may be successfully predicted on the basis of purely stereochemical considerations. A diagrammatic construction of our current conception of the metabolic relationship existing between L-ascorbic acid and the corresponding sugar acids is illustrated in FIGURE 1.

Isolation and Identification of 3-Keto-L-Gulonic Acid

In previous studies concerned with the mechanism of L-xylulose formation



from L-gulonic acid,¹ a partially purified kidney enzyme was described which catalyzed the reactions shown in Equation 1.

Evidence was presented for the accumulation of a small amount of a compound having the properties of a β -ketohexonic acid, although it was not isolated or rigorously characterized at that time. However, it became clear that a two-step reaction was involved, the first of which possessed an absolute requirement for DPN. From this work, it was possible to conclude that the proposed β -keto intermediate is not a precursor of L-ascorbic acid as had been suggested,² but rather that it is an intermediate in the biosynthesis of L-xylulose postulated originally by Touster.³

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The purification procedure, described in detail elsewhere,¹⁰ involving ammonium sulfate fractionation, DEAE cellulose chromatography, and elution from alumina C γ gel, routinely resulted in the recovery of a stable, 100- to 150-fold purified preparation. When this enzyme was tested for specificity, an unexpected pattern of reactivity towards the aldonic acids emerged whereby only those compounds were reactive in which the hydroxyl group of the β -carbon exhibited a levo configuration. In order to check this property in some

TABLE 1
SPECIFICITY OF TPN-L-GULONIC DEHYDROGENASE

Substance	Relative initial rate*	$K_m \times 10^3$
L-Iduronic acid	100	0.55
D-Glucuronic acid	73	1.2
D-Galacturonic acid	35	4.0
D-Mannuronic acid	28	5.7

* The reaction was carried out in a quartz cuvette of 1-cm. light path containing 100 μ moles of phosphate buffer, pH 6.5, 0.10 μ mole TPNH, 0.20 U. of enzyme, and 1.0 μ mole of uronic acid in a total volume of 1.5 ml. Optical density change at 340 $m\mu$ was observed for a 3-min. period on a Beckman D.U. spectrophotometer. The rate observed with L-iduronate was set at 100.

TABLE 2
RELATIVE RATE OF OXIDATION OF VARIOUS β -HYDROXY ACIDS

β -L-Hydroxy acid	Relative activity	β -D-Epimer	Relative activity
L-Gulonic	100	L-Galactonic	0
D-Lyxonic	81	D-Arabonic	0
L-Idonic	59	L-Talonic	0
D-Xylonic	56	D-Ribonic	0
D-Gluconic	49	D-Allonic	0
L-Erythronic	33	D-Threonic	0
L(+) β -Hydroxybutyric	26	D(-) β -Hydroxybutyric	0
D-Threonic	18	D-Erythronic	0
D-Talonic	17	D-Idonic	0
D-Altronic	10	L-Mannonic	0
D-Mannonic	6	D-Altronic	0
D-Ribonic	5	L-Xylonic	0
D-Galactonic	1	D-Gulonic	0
D-Arabonic	0	L-Lyxonic	0
D-Allonic	—	L-Gluconic	0

detail, all of the theoretical isomers of the hexonic, pentonic, and tetrionic acids, with the exception of L-allonic acid, were examined; the result is shown in TABLE 2. In no instance was a positive reaction obtained with a β -D-hydroxy acid. Conversely, upon the demonstration of the unique specificity of this enzyme, it became feasible to test the reversibility of the DPN-linked oxidation reaction (Equation 1) with a number of naturally occurring β -keto acids.

It was readily established that in the presence of the purified enzyme and TPNH, the β -keto function of both acetoacetate and 2,3-diketo-L-gulonate was stereospecifically reduced to L(+) β -hydroxybutyrate and 2-keto-L-gulonate

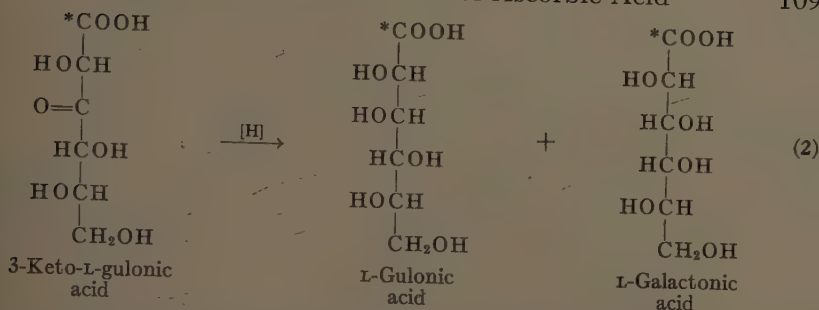
respectively. Significantly, both of the enzymatic reduction products are characterized by the presence of a β -L-hydroxyl group. Since neither of these compounds have been demonstrated previously to participate in mammalian metabolism, unequivocal evidence for their isolation and identification has been obtained and will be described in detail elsewhere.¹⁰ On the basis of the above configurational requirements, it is suggested that the name β -L-hydroxy acid dehydrogenase is more appropriate than the previously used designation of DPN-L-gulonate dehydrogenase.¹

In an attempt to accumulate the immediate product of L-gulonic acid oxidation, a large-scale incubation was carried out. The reaction flask contained 500 μ moles of L-gulonate-1-C¹⁴, 14,000 cpm per μ mole, 500 μ moles pyrophosphate buffer, *pH* 8.5, 500 μ moles sodium pyruvate, 0.2 ml. lactic dehydrogenase, 250 μ moles of cysteine, 10 μ moles DPN, and 50 units of the gulonate oxidizing enzyme in a total volume of 30 ml. The mixture was incubated for 40 min. at 37° C., chilled in an ice bath and, without prior removal of protein, placed on a column containing 200 ml. of pooled Dowex-1-formate. The material was recovered by means of gradient elution with 1 *N* formic acid. A small amount (55 μ moles) of L-xylulose was detected in the water wash of the column. Two subsequent peaks, corresponding to unreacted L-gulonate-1-C¹⁴ (tubes 28 to 31) and to β -keto-L-gulonate (tubes 40 to 60) were revealed by direct plating of aliquots from each tube.

The latter peak, containing 150 μ moles of β -keto-L-gulonate, was pooled, extracted exhaustively with ether to remove formic acid, neutralized to *pH* 6.0 with 1 *N* KOH, and stored frozen at -15° C.

Incubation of this material with the purified dehydrogenase resulted in the rapid oxidation of DPNH. A precise determination of the β -keto-L-gulonate concentration, based upon radioactivity measurements, permitted an accurate evaluation of the stoichiometry of the reaction. At *pH* 6.3, each mole of β -keto-L-gulonate oxidized 1 mole of DPNH in the presence of enzyme. The product of the enzymatic reduction yielded a single spot on paper chromatography, which cochromatographed with synthetic L-gulonolactone in ethyl acetate:pyridine:water:acetic acid (3:5:3:1) and ethyl acetate:acetic acid:water (3:1:3). These solvent systems permitted the ready resolution of this compound from the lactones of galactonic, altronic, talonic, and idonic acid. The metabolic product, eluted from the paper chromatogram was assayed, and the radioactivity determined by direct plating. The specific activity of the reduced product was calculated to be 13,500 cpm per μ mole, which compared closely to the specific activity of 14,000 cpm per μ mole of the starting material.

Final identification of the material recovered from the Dowex column was obtained by isolation of the unique products of the chemical reduction of this β -keto acid. From inspection of Equation 2, it may be seen that of all the theoretically possible isomers of a ketohexonic acid, only 3-keto-L-gulonic acid would be expected to give rise specifically to L-gulonic and L-galactonic acids. Consequently, 10 μ moles of the ketohexonic acid were reduced with potassium borohydride according to methods previously described.⁴ The reduction products, after concentration in 1 *N* HCl to convert the free acids to their respective lactones, were subjected to paper chromatography in ethyl acetate:acetic



acid:water (3:1:3). When examined with AgNO_3 ,¹² two separate spots co-chromatographing with authentic L-gulonolactone and L-galactonolactone were visualized. All the radioactivity was located in these two positions. A more rigorous identification was achieved by elution from the paper, addition of carrier L-gulonolactone and L-galactonolactone to the respective eluates, and repeated recrystallization at constant specific activity.

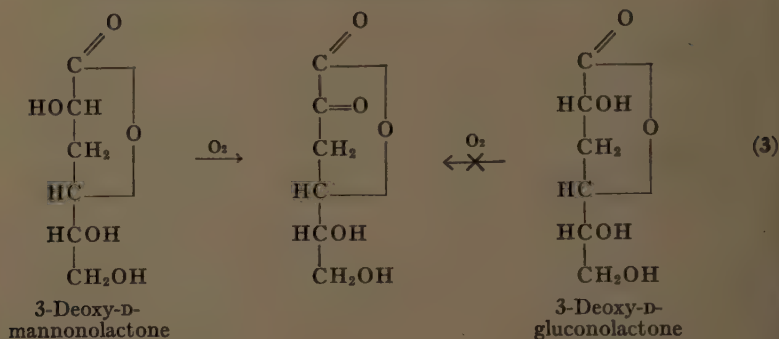
As a consequence of the isolation and final identification of the long postulated 3-keto intermediate, it has now become possible to answer definitively the question as to whether the decarboxylation of this compound, with the resultant formation of L-xylulose, is the result of an enzymatic or nonenzymatic process. James Winkelman, in our laboratory, has successfully purified an enzyme from guinea pig liver acetone powder about 20-fold over the activity of the initial extract and has identified the resulting product as L-xylulose (report in preparation). A more detailed investigation of the properties of this decarboxylase is currently in progress.

Metabolism of L-Ascorbic Acid

In pursuance of an early observation¹² that liver microsomes were the active site of L-ascorbate synthesis from L-gulonolactone, a soluble enzyme was prepared by sonic disruption of rat liver microsomes and purified about five-fold over the activity of the intact particles.¹³ It was reported that the partially purified enzyme possessed a broad specificity requiring, in addition to a lactone ring, a levo configuration of the hydroxyl group on the α -carbon of an hexonic acid.

Following the demonstration of an analogous requirement for the β -hydroxyl group in the case of β -L-hydroxy acid dehydrogenase, further specificity studies on the microsomal enzyme were carried out. TABLE 3 lists the α -epimers of all the four, five, and six carbon sugar acid lactones, with the exception of L-gulonolactone. In every case, the α -L epimer gave rise to ascorbic acid or to an ascorbic acid analogue whereas the α -D compound was completely inert. It is of particular interest to note that 3-keto-L-gulononic acid, available for testing for the first time, did not give rise to L-ascorbic acid.

Actually, it was the realization of this structural specificity that permitted the prediction of 2-keto-L-gulonolactone as the precursor of L-ascorbate. This was accomplished by studies on model compounds susceptible to oxidation at C-2 but incapable of forming ascorbic acid analogues. Thus, as may be seen from Equation 3, 3-deoxy-D-gluconolactone proved to be inert in this system



whereas 3-deoxy-D-mannonolactone was oxidized to form a 2-keto derivative as evidenced by a positive reaction with thiobarbituric acid,¹⁴ as well as with *o*-phenylenediamine.¹⁵ This presumptive evidence by structural analogues has now been buttressed by the report of Chatterjee *et al.*,¹⁶ indicating that the

TABLE 3
CONVERSION OF SUGAR ACID LACTONES TO THE CORRESPONDING
ASCORBIC ACID ANALOGUES

α -L-Hydroxy acid lactone	Relative activity	α -D-Epimer	Relative activity
L-Gulono-	100	L-Idono-	0
L-Galactono-	87	L-Talono-	0
D-Mannono-	68	D-Glucono-	0
D-Altrono-	47	D-Allono-	0
D-Talono-	50	D-Galactono-	0
D-Idono-	65	D-Gulono-	0
L-Glucono-	13	L-Mannono-	0
L-Fucono-	38	—	
D-Glycero-D-ido-heptano-	40	—	
D-Lyxono-	59	D-Xylono	0
L-Xylono-	38	L-Lyxono-	0
D-Arabetono-	22	D-Ribono-	0
L-Ribono-	12	L-Arabetono-	0
L-Erythrono-	15	L-Threono-	0
D-Threono-	23	D-Erythrono-	0

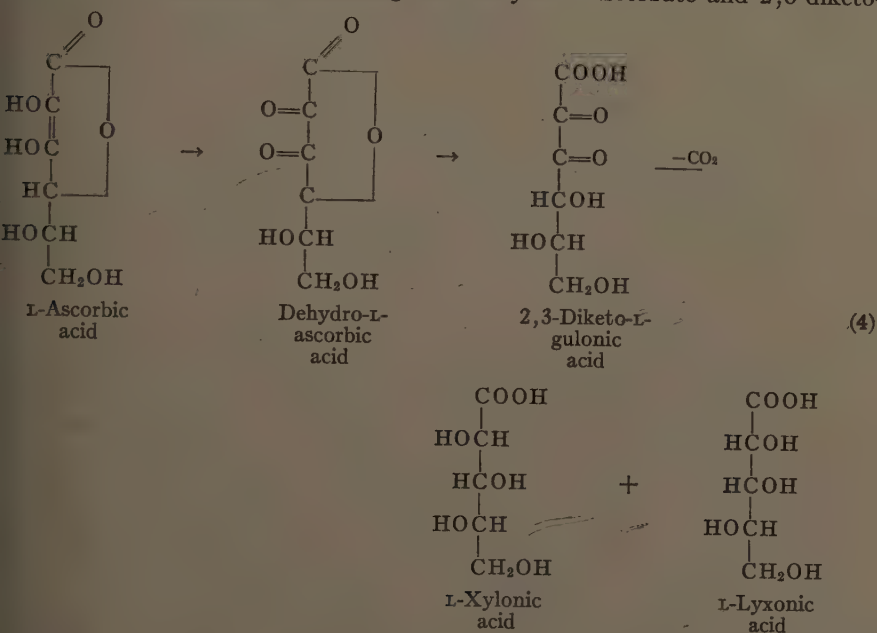
2-Keto-L-gulonic acid }
 3-Keto-L-gulonic acid } All inert.
 L-Gulonic acid }
 D-Glucuronolactone }

quinoxaline derivative of 2-keto-L-gulonic acid has been isolated from a system actively synthesizing L-ascorbate from L-gulonolactone.

Shortly after completion of the original studies on L-ascorbate biosynthesis, our attention was focused on the problem of degradation of the vitamin by mammalian enzymes. The pathway of L-ascorbic acid catabolism is essentially unknown except for the ready oxidation of L-ascorbate to dehydroascorbate and diketo-L-gulonate by a variety of plant and animal tissues. Consequently, the previously mentioned observation that 2,3-diketo-L-gulonate is quantitatively reduced to 2-keto-L-gulonate in the presence of DPNH and the purified β -L-hydroxy acid dehydrogenase, raises the question as to whether this may

be a significant mechanism for the biological utilization of vitamin C. Alternatively, in view of the identification of 2-keto-L-gulonolactone as a precursor in the biosynthesis of L-ascorbate, the enzymatic formation of the free acid may conceivably be viewed as part of a cyclic or sparing mechanism for the maintenance of an adequate ascorbic acid level in the tissues, although *in vivo* studies in rats have failed to demonstrate conversion of uniformly labeled 2-keto-L-gulonate to L-ascorbate.¹⁷ It is interesting to note that the reduction of diketogulonate was found to be experimentally irreversible in contradistinction to all of the other known reactions catalyzed by this enzyme.

Recently an alternate pathway for the catabolism of 2,3-diketo-L-gulonate has been elucidated whereby the pentonic acids, L-xylonic and L-lyxonic acid, have been identified as the products arising from the enzymatic decarboxylation of diketogulonate.¹⁸ Earlier studies on the decarboxylation of L-ascorbate by rat kidney homogenates had shown that factors present in both the soluble and the particulate fraction of the kidney were required for maximal activity, and evidence was presented suggesting that dehydro-L-ascorbate and 2,3-diketo-



-gulonate were intermediates in the decarboxylation of the vitamin.¹⁹ The reaction sequence is illustrated in Equation 4.

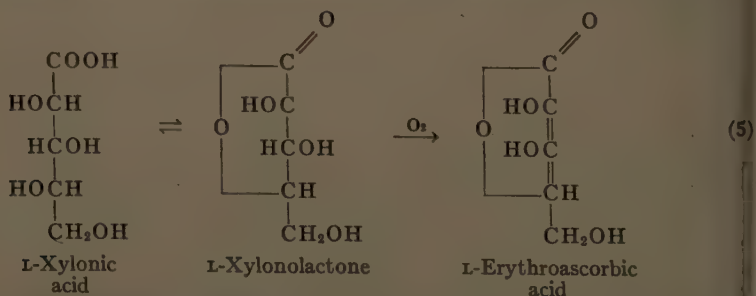
A partially purified enzyme preparation was obtained from the high-speed supernatant fraction of the kidney homogenate. This enzyme readily decarboxylated either dehydroascorbate or diketogulonate but was inert toward ascorbate in the absence of either the microsomal fraction or a small amount of ascorbic acid oxidase. In the case of the former two substrates, the reaction proceeded equally well in air, oxygen, or helium. As described for the other enzymes participating in the metabolism of L-ascorbate, the diketogulonic decarboxylase appeared to possess a broad specificity. In addition to diketo-

L-gulonate, the analogous diketohexonic acids prepared from D-ascorbic acid and D-araboascorbic acid were actively metabolized with the formation of the corresponding pentonic acids.

Isolation and identification of the pentonic acids was accomplished by incubating 75 μ moles of L-ascorbic acid-6- C^{14} (1.73×10^4 cpm per μ mole) with the partially purified enzyme in the presence of Krebs-Ringer phosphate buffer at pH 6.8. A small amount of squash ascorbic acid oxidase was added and the mixture incubated for 30 min. at 37° C. in an atmosphere of oxygen. Following removal of the protein with 4 per cent trichloroacetic acid, the neutralized ether-extracted mixture was adsorbed on a Dowex-1-formate column (1×24 cm.) and elution with 0.01 N formic acid begun. Aliquots of each tube were plated and their C^{14} content determined in a Geiger-Muller proportional counter. Two closely adjacent radioactive peaks were recovered, accounting for approximately 50 per cent of the C^{14} content of the starting material. The more rapidly moving component was identified as L-xylonic acid on the basis of its cochromatography in four solvent systems with an authentic sample, its optical rotation, $[\alpha]_D^{24} = -90^\circ$ C. (as the lactone), and by the isolation of the derivative, xylitol pentoacetate, which was crystallized to constant specific activity in the presence of carrier L-xylonate. Analogous evidence was obtained for the identification of the second component as L-lyxonic acid.

It would appear, as a result of this work, that the major metabolic products of L-ascorbate metabolism in rat kidney are L-xylonic and L-lyxonic acids. In contradistinction to the findings of Chan *et al.*,²⁰ who reported a TPNH stimulated formation of L-xylase by the action of a guinea pig liver supernate upon dehydroascorbic acid, we have been unable to detect the presence of this pentose in the rat kidney system.

Although, at the present time, the physiological significance of none of the above described mechanisms for the catabolism of L-ascorbic acid is clear, we have been impressed by the unusually broad specificity that appears to be characteristic of the entire sequence of reactions involved in ascorbate metabolism. Thus, for example, it was pointed out earlier that all of the aldono-lactones possessing an α -L-hydroxyl group are capable of reacting with L-gulonolactone oxidase to form an ascorbic acid analogue. L-Xylonic acid, a metabolic



product of diketogulonate, possesses such a configuration in the lactone form. From an inspection of Equation 5, it may be seen that in this case the predicted analogue would be L-erythroascorbic acid.

The validity of this prediction was demonstrated in the following manner.

A reaction mixture containing 100 μ moles of L-xylonolactone-1-C¹⁴ (1.6×10^4 cpm per μ mole) was incubated with a rat liver microsomal suspension in 0.1 M phosphate buffer at pH 7.5 for 30 min. at 37° C. in an atmosphere of pure oxygen. The reaction was terminated by the addition of trichloroacetic acid, the protein removed by centrifugation, and the excess trichloroacetic acid extracted with ether. As assayed by the Sullivan method,²¹ 25 μ moles of L-erythroascorbic acid were formed. The neutralized incubation mixture was adsorbed on Dowex-1-formate and recovered by gradient elution with 1 N formic acid. Two radioactive peaks were recovered, the first of which proved to be unreacted L-xylo-1-C¹⁴ acid. The second peak reacted colorimetrically as ascorbic acid, in the Sullivan assay²¹ as well as in the method of Roe and Kuehner,²² although in the latter case the chromogen produced by this analogue was relatively insoluble in the 85 per cent H₂SO₄ and hence not proportional to concentration. Upon paper chromatography in the phenol solvent of Chen *et al.*,²³ the material cochromatographed with synthetic L-erythroascorbic acid and was readily distinguishable from L-xyloascorbic, D-araboascorbic, and D-glucoascorbic acids. Carrier L-erythroascorbic acid was added to the metabolic sample and the mixture recrystallized from acetone and ethyl acetate. The specific activity remained constant during several recrystallizations.

Thus the demonstration of the identity of L-erythroascorbic acid as the *in vitro* metabolic product of liver microsomes on L-xylonolactone provides support for the concept of a cyclic oxidative pathway for the catabolism of vitamin C. At the present time, however, no evidence to document the physiological significance of this sequence is available.

Summary

Four major biochemical aspects of ascorbic acid metabolism in mammalian systems have been described and documented. The reality of 3-keto-L-gulonid as an intermediate in the biosynthesis of L-xylulose has been established by the isolation and identification of this compound. Two new enzymatic mechanisms for the utilization of L-ascorbic acid via diketo-L-gulonate have been presented, a DPNH-linked reduction to form 2-keto-L-gulonate and a carboxylation reaction yielding L-xylo- and L-lyxo- acids. The five-carbon ascorbic acid analogue, L-erythroascorbate, has been demonstrated to be formed enzymatically from L-xylonolactone, in accordance with a proposed cyclic mechanism of vitamin C catabolism. Finally, a clearer appreciation of the unique specificity requirements for each of the purified enzymes participating in this system has been described whereby the reactivity of a given compound may be successfully predicted on the basis of stereochemical considerations.

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METABOLIC INTERACTIONS BETWEEN L-ASCORBIC ACID AND DRUGS

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Interrelationships between the metabolism of L-ascorbic acid and drugs have been the subject of numerous studies. Two aspects of this problem that will be considered here are as follows: (1) The ability of drugs to stimulate the synthesis and metabolism of ascorbic acid, and (2) the ability of ascorbic acid to influence the metabolism of drugs.

Effect of Drugs on Ascorbic Acid Metabolism

Drug-induced ascorbic acid synthesis. Various drugs possessing completely unrelated chemical and pharmacological properties have been shown to stimulate markedly the urinary excretion of L-ascorbic acid in rats.¹⁻³ They include the hypnotics: Chloretone and barbital; the analgesics: aminopyrine and antipyrine; the muscle relaxants: orphenadrine and meprobamate; the antirheumatics: phenylbutazone and oxyphenbutazone; the uricosuric agent, sulfinpyrazone; the antihistaminics: diphenhydramine and chlorcyclizine; and the carcinogenic hydrocarbons: 3-methylcholanthrene and 3,4-benzpyrene.

Of considerable interest is the potent stimulatory effect on ascorbic acid synthesis exerted by the carcinogenic hydrocarbons 3-methylcholanthrene, 1,2,5,6-dibenzanthracene, and 3,4-benzpyrene. The striking effect of a single 10-mg. intraperitoneal injection of 3-methylcholanthrene is shown in Figure 1. For comparison, the effect of a 40-mg. dose of Chloretone is also given. By 6 days after 3-methylcholanthrene administration, the urinary excretion of ascorbic acid was about 70 times greater than the control value and, in fact, during the 19-day period about 140 mg. of the vitamin was recovered in the urine. It will be noted that Chloretone within the first day exerts an effect on ascorbic acid excretion that reaches a peak by the third day and falls to a low value by the fifth day. In contrast, 3-methylcholanthrene exerts no effect for 2 days, but then urinary ascorbic acid excretion increases rapidly and remains elevated for over 18 days.

In another experiment rats were injected intraperitoneally with 10 mg. of the hydrocarbon daily for 3 to 5 days, and the urinary excretion was measured at various intervals thereafter. The urinary excretion of ascorbic acid increased from control values of about 0.3 mg. per day to values of 17 mg. per day by 6 days after the dose. Even by 50 days after administration of the hydrocarbon, the levels of ascorbic acid in urine were still markedly elevated over the control levels. No definite information is available at the present time on the prolonged effect of 3-methylcholanthrene on ascorbic acid synthesis. The possibility that the hydrocarbon remains in the animal by localization in depots is now under investigation.

Turnover rate of ascorbic acid. In order to show further the marked effect

of drugs on ascorbic acid synthesis the turnover of ascorbic acid was determined in rats treated with 3-methylcholanthrene, Chloretone, and pentobarbital; the data are given in FIGURE 2. In these experiments the drugs were given for several days, and then a tracer dose of L-ascorbic acid-1-C¹⁴ was administered intraperitoneally to control or drug-treated rats. The specific activity of ascorbic acid in daily urine samples was determined at various times after the dose of the labeled compound.⁴ In each case, 24 hours were allowed to lapse before the first specific activity value was determined in order to permit sufficient time for equilibration of the radioactive ascorbic acid with nonlabeled ascorbic acid in the animal. The specific activity values obtained have been

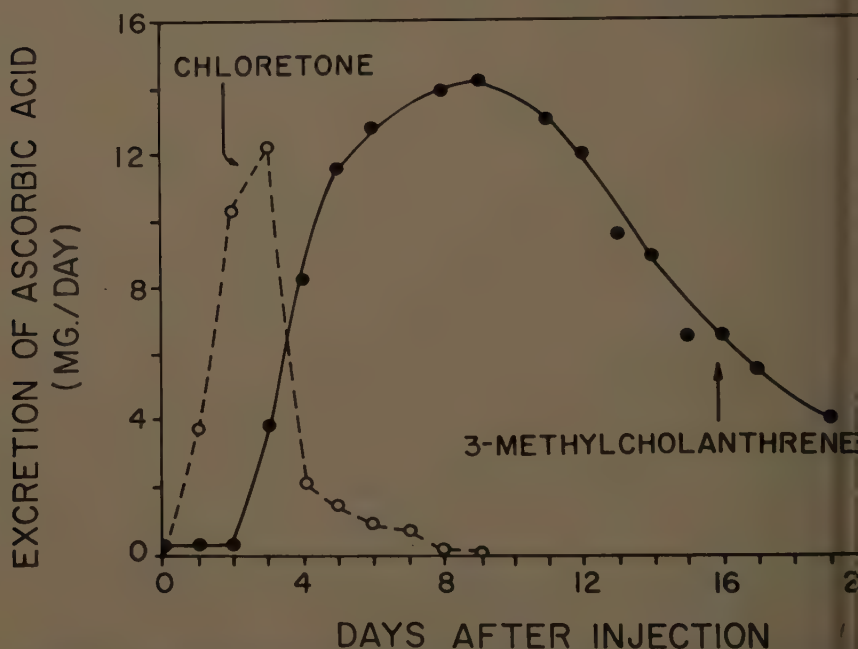


FIGURE 1. Urinary excretion of L-ascorbic acid by rats following a single intraperitoneal injection of 3-methylcholanthrene (10 mg.) or Chloretone (40 mg.).

plotted on a log scale at the mid-point of each urine collection period. The data show that 3-methylcholanthrene, Chloretone, and pentobarbital markedly shorten the half life of ascorbic acid in the rat. For example, in the normal rat the half life is 3 days; in the 3-methylcholanthrene-treated animal it is only 0.8 day.

From these data it is possible to calculate the body pool and turnover rate of ascorbic acid;⁴ these values are given in TABLE 1. In untreated rats, the body pool of ascorbic acid is 10.7 mg. per 100 gm. body weight, while in drug-treated animals the body pool of ascorbic acid about doubles. The turnover rate of ascorbic acid (the amount synthesized and metabolized or excreted each day) in control rats was about 2.6 mg. per 100 gm. rat per day. This value was increased about 10-fold by pretreating the rats with Chloretone or

ethylcholanthrene, and about 5-fold by pretreating the animals with pentobarbital.

The amount of ascorbic acid excreted daily in the urine did not account for of the ascorbic acid synthesized each day. For example, in control rats out 1.8 mg. ascorbic acid per 100 gm. body weight was metabolized each day, whereas in the pentobarbital-, 3-methylcholanthrene-, or Chloretone-treated rats, this value increased to about 10 mg. per 100 gm. body weight per day

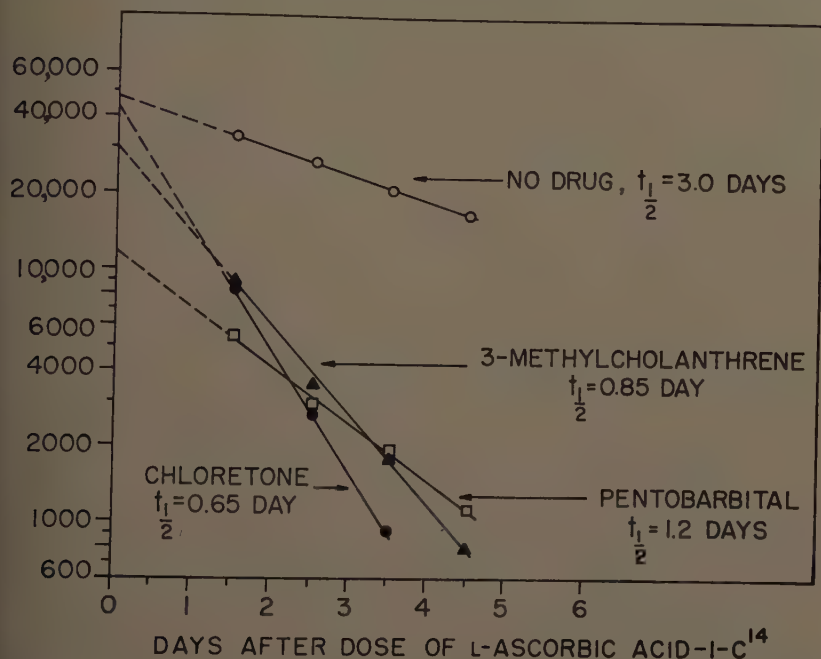


FIGURE 2. Turnover rate studies with L-ascorbic acid in drug-treated rats. Pentobarbital (30 mg.) or Chloretone (45 mg.) was administered orally for several days prior to administration of L-ascorbic acid-1-C¹⁴. 3-Methylcholanthrene (10 mg.) was injected intraperitoneally daily for 4 days; the L-ascorbic acid-1-C¹⁴ 5 days later.

TABLE 1
EFFECT OF DRUGS ON ASCORBIC ACID METABOLISM IN THE RAT*

Drug pretreatment	Body pool of AA (mg./100 gm.)	Turnover rate of AA (mg./100 gm./day)	Excretion of AA (mg./100 gm./day)	Metabolism of AA (mg./100 gm./day)
Control	10.7	2.6	0.40	2.2
Chloretone	19.2	21.5	10.2	11.3
3-methylcholanthrene	22.5	19.0	7.1	11.9
Pentobarbital	19.6	11.6	2.4	9.2

Pentobarbital (30 mg.) or Chloretone (45 mg.) was administered orally for 4 to 7 days prior to the administration of L-ascorbic acid-1-C¹⁴. Methylcholanthrene (10 mg.) was injected intraperitoneally daily for 4 days, and the L-ascorbic acid-1-C¹⁴ was administered 5 days later. All values are given on a milligram per 100-gm. body weight basis.

(TABLE 1). Thus administration of drugs not only leads to increased urinary excretion of the vitamin, but also to a marked increase in its metabolic breakdown. Examination of the respiratory CO_2 at various intervals after administration of L-ascorbic acid-1- C^{14} showed that significant amounts of C^{14} were excreted by this route. In addition, evidence was found for the excretion of considerable amounts of labeled metabolites of ascorbic acid in the urine of drug-treated rats.

Agents inhibiting ascorbic acid synthesis. Administration to rats of adenosine triphosphate (ATP) has been reported to inhibit the synthesis of ascorbic acid.⁵ Experiments were carried out in our laboratory to investigate further this inhibitory effect of ATP. Three rats were given 25 mg. of Chloretone each by stomach tube daily for 7 days. In addition to this dosage of Chloretone, the animals then received by intramuscular injection 120 mg. of ATP daily, divided into 2 equal doses. The excretion of L-ascorbic acid dropped from an average value of 41 ± 2.0 mg./day, while the animals received only the Chloretone, to an average value of 15 ± 2.0 mg./day, when they received both Chloretone and ATP. Increasing the dosage of ATP to 150 mg./day, divided into 3 equal doses, did not decrease further the excretion of L-ascorbic acid.

The alkaloid lycorine has been reported to inhibit the synthesis of L-ascorbic acid in rats.⁶ In order to test this, we gave each of 3 rats 20 mg. of Chloretone daily by stomach tube for 3 days. The animals then received 6 mg. of lycorine divided in two doses by subcutaneous injection, along with the dosage of Chloretone. L-Ascorbic acid excretion dropped from an average control value of 21.6 ± 3.0 mg. to 14.2 ± 1.7 mg. when the animals received both Chloretone and lycorine. The results indicate that lycorine had a distinct but far from complete effect in inhibiting L-ascorbic acid synthesis in Chloretone-treated rats.

Ascorbic acid synthesis via the glucuronic acid pathway. Administration of drugs to rats results in increased metabolism of glucose through the glucuronic acid pathway shown in FIGURE 3. The reactions of this pathway have been reviewed recently by Touster,⁷ Burns,⁸ Strominger,⁹ and Burns and Conney.¹⁰ According to this scheme, D-glucose is oxidized to D-glucuronic acid through the intermediate formation of uridine nucleotides. The D-glucuronic acid undergoes reduction to L-gulonic acid, which serves as the precursor of either ascorbic acid or L-xylulose.

Evidence that drugs stimulate the glucuronic acid pathway comes from the finding that administration of barbital or Chloretone to rats stimulates markedly the conversion of D-glucose-1- C^{14} or D-galactose-1- C^{14} to labeled D-glucuronic acid, L-gulonic acid, and L-ascorbic acid.^{3,11,12} This effect of barbital to stimulate glucose and galactose metabolism through the glucuronic acid pathway is shown in TABLE 2. Rats were given 100 mg. oral doses of barbital daily for several days and were then injected with D-glucose-1- C^{14} or D-galactose-1- C^{14} . Urine was collected for 24 hours, and the amount of radioactivity in isolated free D-glucuronic acid as well as in L-gulonic acid and L-ascorbic acid was determined. It is seen here that control rats convert less than 0.05 per cent of the D-glucose or D-galactose to D-glucuronic, L-gulonic, or L-ascorbic acids, and that considerable conversion took place in barbital-treated rats.

Additional experiments were carried out on the effect of 3-methylcholanthrene on the synthesis of L-ascorbic acid from D-galactose, and the results are shown in TABLE 3. In these experiments D-galactose-1-C¹⁴ was administered to rats, and the incorporation of C¹⁴ into urinary D-glucuronic, L-gulonic, and

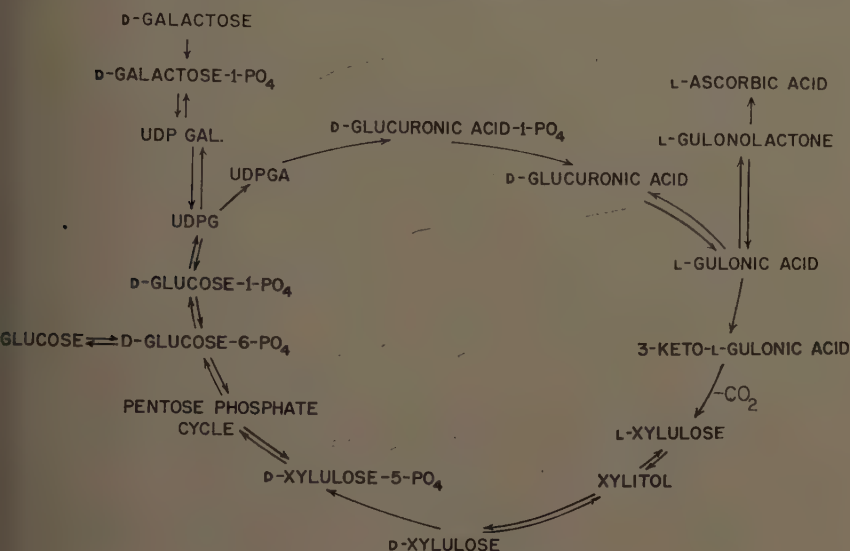


FIGURE 3. Glucuronic acid pathway.

TABLE 2
CONVERSION OF D-GLUCOSE-1-C¹⁴ AND D-GALACTOSE-1-C¹⁴ TO GLUCURONIC ACID PATHWAY
INTERMEDIATES EXCRETED IN RAT URINE

Precursor	Per cent of administered C ¹⁴ in		
	D-Glucuronic acid	L-Gulonic acid	L-Ascorbic acid
<i>Control rats</i>			
Glucose-1-C ¹⁴	<0.03	<0.03	<0.02
Galactose-1-C ¹⁴	<0.05	<0.05	<0.05
<i>Barbital-treated rats*</i>			
Glucose-1-C ¹⁴	0.41	0.19	0.19
Galactose-1-C ¹⁴	1.49	0.65	0.77

* Rats were pretreated with 100 mg. of barbital orally for 7 days prior to the experiment.

ascorbic acids was measured. It will be noted that there was a considerable increase in the ability of 3-methylcholanthrene-treated rats to metabolize galactose-1-C¹⁴ to labeled L-gulonic and L-ascorbic acids beyond that observed in control rats. These data are similar to those obtained with barbital-treated rats (TABLE 2). However, in contrast to the extensive incorporation of galactose-1-C¹⁴ into D-glucuronic acid in barbital-treated rats, considerably

less was noted in the 3-methylcholanthrene-treated rats. Other studies have shown that barbital increased markedly the level of free D-glucuronic acid in urine, whereas little or no increased excretion was observed following 3-methylcholanthrene treatment. The possibility that 3-methylcholanthrene may stimulate the further metabolism of D-glucuronic acid as well as its formation is being investigated.

Evidence for the stimulatory effect of drugs on the glucuronic acid pathway (FIGURE 3) also comes from the earlier studies of Enklewitz and Lasker¹³ in human subjects with essential pentosuria. These subjects possess a metabolic defect that prevents the further metabolism of L-xylulose. When aminopyrine or antipyrine, drugs that stimulate ascorbic acid production, were administered to these subjects, the urinary excretion of L-xylulose was markedly elevated.

The exact mechanism by which drugs stimulate the formation of L-ascorbic acid via the glucuronic acid pathway is not known. The possibility that a renal mechanism is involved has been ruled out since drugs can stimulate ascorbic acid formation in nephrectomized rats.³ The formation of a glucuronide

TABLE 3
EFFECT OF 3-METHYLCHOLANTHRENE ON METABOLISM OF GALACTOSE-1-C¹⁴ TO GLUCURONIC ACID PATHWAY INTERMEDIATES EXCRETED IN RAT URINE

Pretreatment	Per cent of administered galactose-1-C ¹⁴ in		
	D-Glucuronic acid	L-Gulonic acid	L-Ascorbic acid
None	<0.05	<0.05	<0.05
3-Methylcholanthrene*	0.14	0.61	0.56

* 10 mg. of 3-methylcholanthrene was injected intraperitoneally into rats daily for 4 days. Galactose-1-C¹⁴ was administered 5 days later.

ide by the drug is not required since barbital, a drug that stimulates markedly the production of free glucuronic acid and also L-gulonic and L-ascorbic acids, is excreted unchanged in the urine.¹¹ Several compounds such as borneol and α -naphthol, which are extensively conjugated as glucuronides, do not stimulate in rats the synthesis of L-ascorbic acid or its precursor L-gulonic acid.

In vitro studies. The possibility that pretreatment of rats with Chloretone may increase the activity of liver enzymes required for ascorbic acid synthesis was investigated. It was found that increased metabolism of D-galactose-1-C¹⁴ to free D-glucuronic acid occurred in liver homogenates derived from Chloretone-treated rats (TABLE 4). In this experiment, 300 to 325-gm. rats were given 40 mg. of Chloretone orally each day for 7 days. On the following day liver homogenates were prepared and incubated with D-galactose-1-C¹⁴ in the presence of adenosine triphosphate (ATP), uridine diphosphoglucose (UDPG), MgCl₂, diphosphopyridine nucleotide (DPN), and nicotinamide.¹² Formation of radioactive D-glucuronic acid was determined by a specific carrier dilution procedure. It is seen here that D-galactose is metabolized to D-glucuronic acid 3 to 4 times more readily by liver homogenates obtained from Chloretone-treated rats than by those from control rats.

More detailed studies on the effect of Chloretone on individual enzymatic

steps involved in ascorbic acid synthesis were then carried out. In these experiments 250- to 300-gm. rats were given 40 mg. of Chloretone orally daily for 7 days. They were killed on the following day, and the livers were used

TABLE 4
EFFECT OF CHLORETONE ON THE CONVERSION OF GALACTOSE-1-C¹⁴ TO D-GLUCURONIC ACID
IN RAT LIVER HOMOGENATE

Rats	Per cent conversion to D-glucuronic acid
Control	0.21
Control	0.18
Chloretone*	0.68
Chloretone*	0.88
Chloretone*	0.77

* Rats were pretreated with 40 mg. of Chloretone orally for 7 days prior to the experiment.

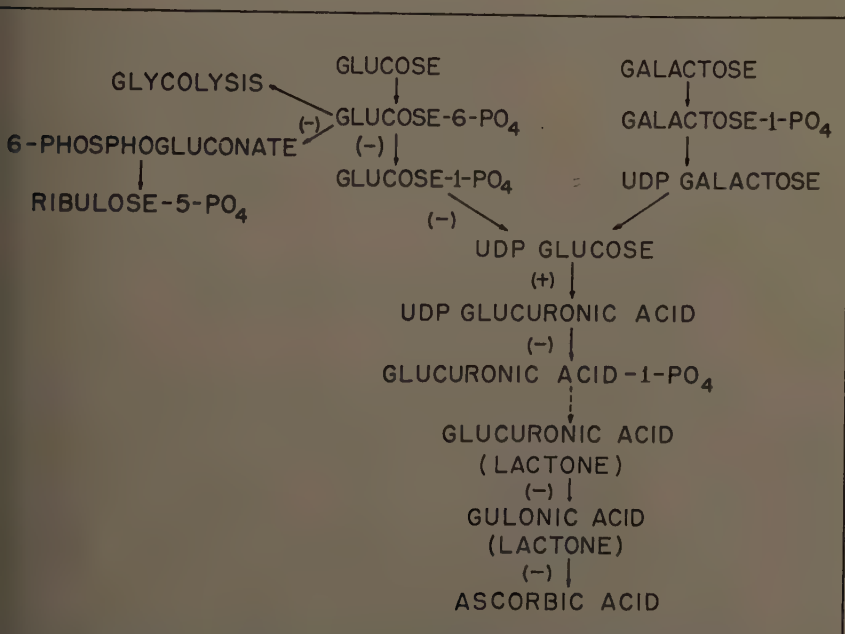


FIGURE 4. Effect of Chloretone pretreatment on liver enzymes involved in ascorbic acid synthesis. Chloretone (40 mg.) was administered orally for 7 days prior to the experiment.

carry out studies on the various enzymatic reactions shown in FIGURE 4. The effect of Chloretone pretreatment on the activities of these liver enzymes is indicated by a plus or minus sign. Chloretone pretreatment did not stimulate the enzyme system that converts D-glucuronolactone to L-gulonolactone or the enzyme system that converts L-gulonolactone to L-ascorbic acid. In fact, the enzyme system that converts L-gulonolactone to L-ascorbic acid was depressed about 50

per cent. These results are consistent with observations that drugs do not accelerate the *in vivo* conversion of D-glucuronolactone or L-gulonolactone to L-ascorbic acid.¹⁴ It therefore appears that drugs that stimulate ascorbic acid synthesis act at some step prior to D-glucuronic acid. The effects of Chloretone pretreatment on glycolytic enzymes, hexokinase, or glucose-6-phosphatase have not yet been investigated. The levels of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucomutase, and UDPG pyrophosphorylase were not significantly changed by Chloretone treatment. It was found, however, that the level of UDPG dehydrogenase, the system required to convert uridine diphosphoglucose to uridine diphosphoglucuronic acid (UDPGA), was elevated about 100 per cent in Chloretone-treated rats. The activity of the enzyme system in liver particulate that metabolizes UDPG to D-glucuronic acid-1-phosphate was not stimulated. Only trace amounts of D-glucuronic acid were formed when UDPGA was incubated with livers from either control or Chloretone-treated rats.

TABLE 5
EFFECT OF PRETREATMENT OF RATS WITH CHLORETONE ON THE ACTIVITY
OF UDPG DEHYDROGENASE

Rats	Relative enzyme activity			
	Fresh	Aged*	Aged* with UDPG	Aged* with UDPGA
Control	100	0	33	77
Chloretone-treated†	185	125	—	168

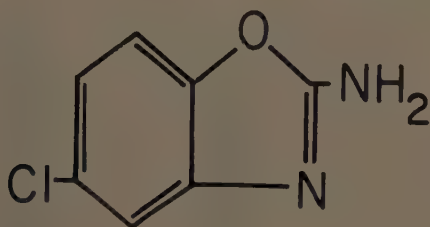
* The enzyme preparation was allowed to stand at 4° C. for 7 days.

† Rats were pretreated with 40 mg. of Chloretone orally for 7 days prior to the experiment.

Of the enzymes studied, only UDPG dehydrogenase levels were increased by Chloretone administration; therefore, this enzyme system was investigated in more detail. A number of the results are presented in TABLE 5. The interesting observation was made that upon standing at 4° C. the UDPG dehydrogenase activity in the high-speed liver supernatant fraction of control rats decreased in activity. Thus little or no UDPG dehydrogenase activity was observed in enzyme preparations that had been allowed to age at 4° C. for 5 to 7 days. On the other hand, similar enzyme preparations obtained from Chloretone-treated rats decreased only slightly in UDPG dehydrogenase activity when aged. This decrease in UDPG dehydrogenase with aging at 4° C. was prevented by allowing the enzyme to age in the presence of added UDPG, UDPGA, or uridine triphosphate (UTP), but not with added ATP or Chloretone. These studies raise the interesting possibility that Chloretone administration may accelerate *in vivo* the metabolism of UDPG to UDPGA by stabilizing UDPG dehydrogenase. This could possibly occur by increasing the level of uridine nucleotides in liver. It is not known at present whether the effect of Chloretone in stimulating the synthesis of glucuronic acid may be explained by a stimulation and stabilization of UDPG dehydrogenase. Further studies are

progress to determine the relevance of this enzymatic step in ascorbic acid synthesis.

Effect of drugs on drug-metabolizing enzymes. The results given here show that various drugs stimulate the metabolism of D-glucose and D-galactose to ascorbic acid through the glucuronic acid pathway. Recent studies indicate that drug administration exerts other biochemical effects. One effect studied in our laboratory is the ability of drugs to stimulate the activity of drug-metabolizing enzymes. It was found that drugs potent in stimulating ascorbic acid synthesis in rats are also potent in stimulating the activity of drug-metabolizing enzymes in liver microsomes.^{2,15} Examples of drugs that exert these effects are 3,4-benzpyrene, 3-methylcholanthrene, Chloretone, phenobarbital, barbitol, phenylbutazone, aminopyrine, orphenadrine, and chlorcyclizine. Examples of drug-metabolizing enzymes that are increased by administration of these inducer drugs include those enzymes that metabolize hexobarbital, pentobarbital, aminopyrine, phenylbutazone, 3,4-benzpyrene, and zoxazolamine.



ZOXAZOLAMINE

FIGURE 5.

Studies with zoxazolamine (structure shown in FIGURE 5) illustrate the effect of drugs on a drug-metabolizing enzyme. Zoxazolamine, a muscle-relaxant drug, is hydroxylated in the 6-position by liver microsomes to yield a pharmacologically inactive metabolite.¹⁶ In the experiment shown in FIGURE 6, 40-gm. rats were injected intraperitoneally with 75 mg./kg. of phenobarbital daily for 4 days or with 25 mg./kg. of 3,4-benzpyrene 24 hours before killing the animals and isolating their liver microsomes. It was found that pretreatment of the rats with these compounds markedly increased the activity of the liver microsomal enzyme system that metabolizes zoxazolamine. The increased enzyme activity was correlated with a shortened duration of zoxazolamine paralysis. The duration of action of zoxazolamine in control rats was 730 min., while in phenobarbital- or 3,4-benzpyrene-treated rats it was 102 min. or 17 min., respectively. Several lines of evidence indicate that these stimulators of enzyme activity actually induce the synthesis of drug-metabolizing enzymes.^{15,17,18} For instance, the decreased activity of drug-metabolizing enzymes observed after pretreatment with phenobarbital or 3,4-benzpyrene can be completely blocked by administration of certain amino acid antagonists such as ethionine, an agent that inhibits protein synthesis. The effect of ethionine in blocking enzyme induction

can be completely overcome by the simultaneous administration of the amino acid methionine. In view of the effect of drugs in inducing the synthesis of drug-metabolizing enzymes, it is possible that drugs may also stimulate ascorbic acid synthesis by inducing the synthesis of enzymes of the glucuronic acid pathway. In this connection it is of interest to note that administration of methionine to rats has been recently reported to block the ability of barbiturates and 3-methylcholanthrene to increase ascorbic acid synthesis.¹⁹

The mechanism by which drugs can both stimulate ascorbic acid biosynthesis and increase the activity of drug-metabolizing enzymes in liver microsomes is not known, but these effects appear to represent adaptive responses to drug administration that occur in the absence of the adrenal gland.^{11,17} We have

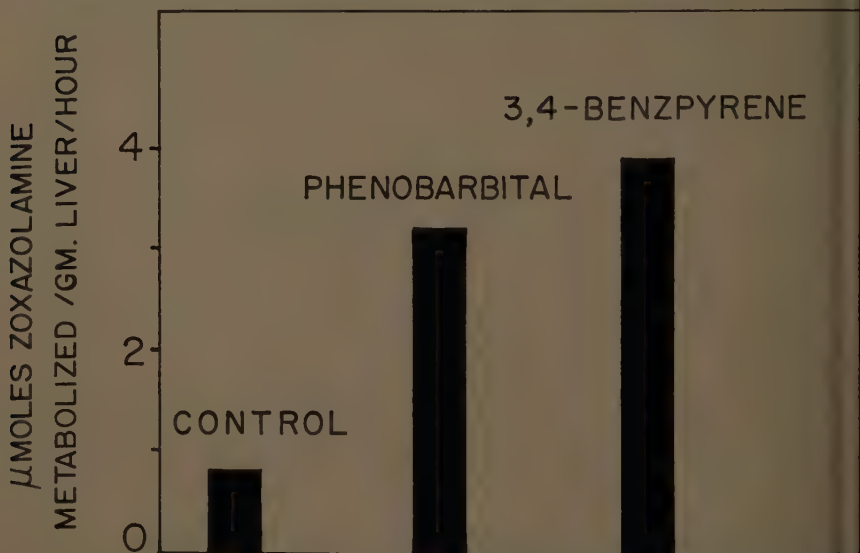


FIGURE 6. Stimulatory effect of phenobarbital and 3,4-benzpyrene administration on the activity of xozazolamine hydroxylase in rat liver microsomes. Enzyme assays were carried out in the presence of a system that generated reduced triphosphopyridine nucleotide.

been intrigued by the possibility that both of these responses to drugs may represent adaptations that are beneficial to the animal. Certainly, it is apparent that increased activity of drug-metabolizing enzymes in response to drug administration can result in accelerated drug detoxication. The effect of drugs to stimulate the glucuronic acid pathway (FIGURE 3) leads to increased production of UDPGA, D-glucuronic acid, and L-ascorbic acid. UDPGA is necessary for the conjugation of drugs as glucuronides. D-Glucuronic acid, when administered as its lactone, has been reported to protect animals from various toxic agents.²⁰ The possibility that L-ascorbic acid may be involved in the metabolism of drugs is pointed out in the following section.

Effect of Ascorbic Acid on Drug Metabolism

Reports have appeared in the literature indicating that vitamin C-deficient guinea pigs are unusually sensitive to various drugs. For example, Richman

his co-workers reported that vitamin C-deficient guinea pigs were more sensitive than normal guinea pigs to pentobarbital and to procaine.^{21,22} In other studies Axelrod and his co-workers²³ have shown that the rate of *in vivo* hydroxylation of acetanilid and aniline was decreased in vitamin C-deficient guinea pigs.

Recent studies in our laboratory have indicated that vitamin C-deficient guinea pigs are more sensitive to the muscle-relaxant drug zoxazolamine than normal guinea pigs. The increased sensitivity toward zoxazolamine can be explained by decreased activity of the enzyme system in liver microsomes that metabolizes this drug (TABLE 6). In these experiments the guinea pigs were placed on a scorbutogenic diet. One half of the animals were given daily doses of 10 mg. of ascorbic acid orally; the other half were given glucose. The animals were maintained on the scorbutogenic diet for 10 to 14 days, and at this time they showed no obvious signs of scurvy. It may be seen that the duration of zoxazolamine paralysis in guinea pigs receiving ascorbic acid supple-

TABLE 6
EFFECT OF ASCORBIC ACID DEFICIENCY IN GUINEA PIGS ON DURATION OF ZOXAZOLAMINE PARALYSIS AND ON THE ZOXAZOLAMINE-METABOLIZING ENZYME SYSTEM IN LIVER MICROSOMES*

Diet	Duration of zoxazolamine paralysis (min.)	<i>In vitro</i> metabolism (μg. zoxazolamine metabolized)
Ascorbic acid-supplemented†	156 ± 41 (10)	36 ± 12 (15)
Ascorbic acid deficient	309 ± 27 (20)	12 ± 8 (15)

*The duration of zoxazolamine paralysis was determined after the intraperitoneal injection of 100 mg./kg. of zoxazolamine. The *in vitro* enzyme assays were carried out by incubating liver microsomes from 375 mg. of liver with 100 μg. of zoxazolamine for 15 min. in the presence of a system that generated reduced triphosphopyridine nucleotide.¹⁵ The number of animals used are indicated in parentheses.

†The animals received 10 mg. of ascorbic acid orally each day.

ment was 156 min., whereas the guinea pigs that did not receive the vitamin were paralyzed for 309 min. The increased sensitivity of vitamin C-deficient guinea pigs was paralleled by decreased activity of the liver microsomal enzyme system that metabolizes zoxazolamine. Thus liver microsomes from ascorbic acid-supplemented guinea pigs metabolized an average of 36 μg. of zoxazolamine, while microsomes from the vitamin C-deficient animals metabolized an average of only 12 μg. of zoxazolamine. The addition of ascorbic acid *in vitro* to microsomes obtained from vitamin C-deficient guinea pigs did not increase the activity of this enzyme system. It should be emphasized that the decreased activity of the zoxazolamine-metabolizing enzyme system occurs at an early stage in vitamin C deficiency; this is observed before gross deficiency symptoms such as loss of weight and hair and severe joint manifestations are evident.

A model system consisting of L-ascorbic acid, ferrous ion, ethylenediamine-acetic acid, and oxygen has been shown to catalyze the hydroxylation of aromatic compounds as acetanilid, antipyrine, aniline, anthranilic acid, kynurenine to yield products identical with those formed in the body.²⁴⁻²⁶ The importance of this system for the metabolism of drugs in the animal remains to be established.

Summary

The administration of various drugs to rats stimulates L-ascorbic acid synthesis from D-glucose and D-galactose through the glucuronic acid pathway. Increased metabolism of L-ascorbic acid also occurs in drug-treated rats. Although the enzymatic basis for increased ascorbic acid synthesis is not known, drugs appear to act on some step before the formation of D-glucuronic acid. The effect of Chloretone pretreatment to increase the activity of UDPG dehydrogenase in liver has been pointed out. It is of interest that drugs that stimulate ascorbic acid synthesis also induce the synthesis of drug-metabolizing enzymes in liver microsomes. It is possible that the effect of drugs on ascorbic acid synthesis also reflects induced enzyme synthesis.

Results presented here show that vitamin C deficiency makes guinea pigs more sensitive to the muscular-relaxant drug zoxazolamine by decreasing the activity of the liver microsomal enzyme system required for the metabolism of zoxazolamine.

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SOME ASPECTS OF THE METABOLISM OF ASCORBIC ACID IN RATS*

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Remarkable progress has been recorded during the past few years in elucidation of pathways involved in the biogenesis of ascorbic acid, in reactions involved in its catabolism, and in the metabolism of related compounds. While studies *in vivo* can never hope to reveal the fine detail of enzymic mechanisms, it is nevertheless true that recent progress has been made possible by earlier findings with whole animals. It is anticipated that the present modest study will follow the traditional pattern. In this report, which concerns aspects of the metabolism of ascorbic acid in rats, more problems are raised than answered. Current investigations of simplified systems should remedy this unsatisfactory condition.

It has been found that ACTH (corticotropin) results in marked depression of radioactivity in respiratory carbon dioxide originating from C-1 of carboxyl-labeled ascorbic acid. In further investigation of hormonal effects, it was noted that hypophysectomy caused an abrupt decrease of urinary excretion, an increase in the half life, and a decrease in size of the body pool of ascorbic acid. Urinary excretion of glucuronic acid was less markedly depressed, and differences disappeared entirely over a period of time. A multiple hormonal deficiency may be responsible for the defect in ascorbic acid synthesis. However, although thyroidectomy did not affect the half life or body pool of ascorbic acid and neither ACTH nor cortisone acetate affected the urinary excretion of either ascorbic acid or glucuronic acid, growth hormone did result in increased excretion of ascorbic acid. The latter hormone did not affect excretion of glucuronic acid.

When Chloretone was administered to hypophysectomized rats, a prompt increase in excretion of ascorbic acid was effected. Although urinary excretion of ascorbic acid was maintained substantially above the level of that of untreated control rats during pretreatment of hypophysectomized rats with growth hormone, the differences largely disappeared during chloretonization. Furthermore, differences in this respect between hypophysectomized and intact rats, which were even more marked, greatly diminished during treatment with Chloretone. Urinary glucuronic acid also increased in all cases, but increases were much smaller than those of ascorbic acid, and the concentration rose after accelerated excretion of ascorbic acid was well under way. The results tend to indicate an especially important effect on one phase of the synthesis of ascorbic acid following glucuronic acid, as well as a stimulatory action of growth hormone and of Chloretone above that provided by an increase in formation of glucuronic acid.

* The investigation reported in this paper was supported in part by Research Grant C-394 from the National Cancer Institute, Public Health Service, Bethesda, Md., and by Research Grant G-4436 from the National Science Foundation, Washington, D.C.

Experimental

Ascorbic-1-C¹⁴ acid was prepared as previously described.¹ Injected doses were close to 1.0 mg. (3.75 μ c.) in each case. Analyses for total ascorbic acid were performed by the method of Roe and Kuether² as modified by Lowry *et al.*,³ and, for differential determination of reduced and oxidized ascorbic acid, by the method of Roe *et al.*,⁴ which was slightly modified. Glucuronic acid was determined by the method of Maughan *et al.*⁵ For metabolism experiments, equipment described elsewhere⁶ was employed. Samples of respiratory carbon dioxide and urine were prepared for counting on paper disks by standard procedures. Urine specimens were collected in iced flasks containing a solution of oxalic acid.

The specific activity of total body ascorbic acid was determined as follows. The entire carcass was thoroughly ground in a meat grinder through successively finer sieves, after removal of contents of stomach and intestine. The mass was added to 4 volumes (based on the weight of the tissues) of 10 per cent trichloroacetic acid that had been saturated with H₂S at 0° C., and an additional stream of H₂S was passed through the mixture for 15 min. After 24 hours at 2 to 3° C. with occasional shaking, the supernate was removed by decantation with suction, using a tight-fitting plug of glass wool in the funnel. Remaining suspended material was removed by filtration through a fluted filter. From a portion of the supernate, H₂S was removed for determination of ascorbic acid. To the remainder, 300 to 400 mg. of carrier ascorbic acid was added.

In initial experiments it was found that no dehydroascorbic acid or diketogluconic acid remained in the supernate after 24 hours, presumably because the former had been largely reduced and the latter decomposed. Consequently, analyses for total ascorbic acid were adequate for determination of the extracted ascorbic acid. Isolation of ascorbic acid from the remaining supernate was made with Amberlite IR-45 ion exchange resin in the acetate form and with Amberlite IR-120 in the acid form. The supernate was thoroughly extracted with ether to remove H₂S and excessive trichloroacetic acid. After removal of residual ether under vacuum, the solution was passed through a column of anion exchanger directly into the anion exchanger. Following washing with water, the column containing the cation resin was removed, and ascorbic acid was eluted from the anion exchanger with 1 *N* formic acid. Either the 2,4-dinitrophenyl osazone of ascorbic acid or crystalline material may be readily obtained from the eluate.

Hypophysectomized and intact male rats of the Sprague-Dawley strain were used.* Pair-feeding of animals was considered undesirable because of the potential interference of inanition in the intact groups and in hypophysectomized rats receiving growth hormone. No special diets designed to enhance excretion of ascorbic acid were provided. Growth hormone† was administered to the appropriate groups of rats in daily intraperitoneal doses of 150 γ , which support more than half-maximal growth. Rats so treated attained weights of approximately 170 to 180 gm., as compared with weights in excess of 450 gm. reached

* Obtained from Hormone Assay, Inc., Chicago, Ill.

† Somar, The Armour Laboratories, Kankakee, Ill.

by intact male adults. ACTH was injected intraperitoneally in the dosage indicated in the text, and cortisone acetate* subcutaneously in doses of 5 mg. per 100 gm. of body weight. Chloretone was homogenized in evaporated milk and given daily by stomach tube in doses of 9 mg. per 100 gm. of body weight.

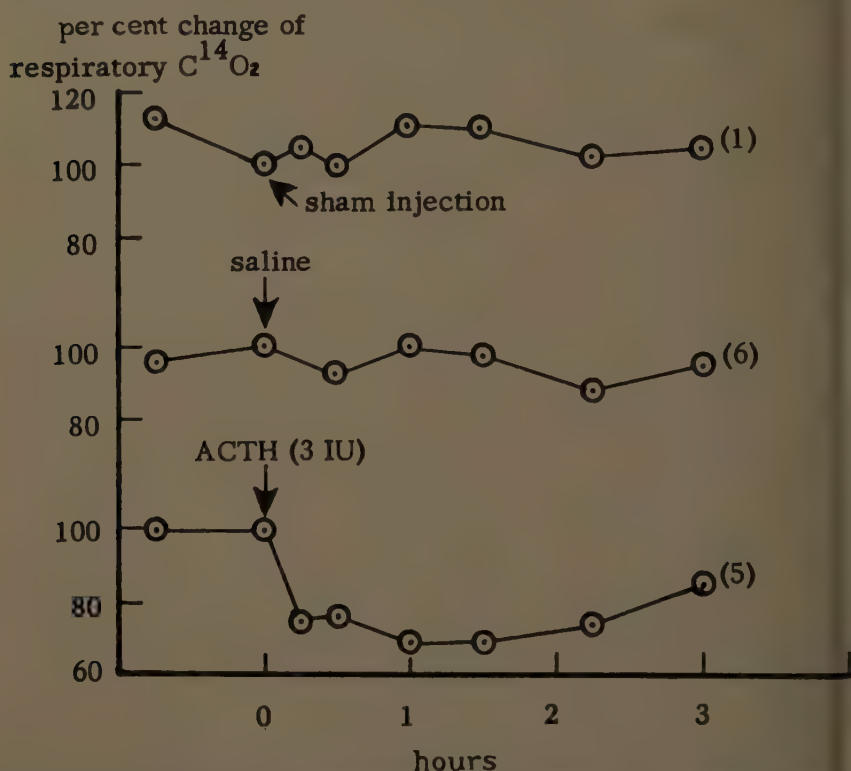


FIGURE 1. Effect of ACTH on respiratory $C^{14}O_2$. These rats had received ascorbic C^{14} acid 16 hours prior to the start of the experiment. The number of animals in each group is indicated in parentheses.

Results and Discussion

In an earlier publication, results were presented that indicated that the steady-state half life of ascorbic acid in the guinea pig was not altered by moderately severe scurvy or by administration of lethal doses of diphtheria toxin. For the present purposes, it may be noted that failure of physiological stress to affect the rate of catabolism of ascorbic acid under the conditions of the experiments was especially surprising. Nevertheless, in the hypophysectomized rat, administration of ACTH in adrenal weight-maintaining quantities produced a significant although transient decrease in excretion of radioactive carbon dioxide derived from carboxyl- C^{14} -labeled ascorbic acid (FIGURE 1). This effect, absent in control rats receiving injections of saline, was quite un-

* Cortogen acetate, Schering Corporation, Bloomfield, N.J.

expected. Since it was not possible to obtain urine specimens reliably equaling periods of collection of respiratory gases, however, these experiments cannot be considered conclusive. They did indicate that hormonal factors might play a role in the metabolism of ascorbic acid; hence the problem was pursued further. In the subsequent comparison of hypophysectomized and intact rats, it was found that there was a prompt increase in the half life of ascorbic acid following hypophysectomy. The data of TABLE 1 show that measured values for the half life for both groups of animals exhibited considerable dispersion and overlap. The difference of the means is significant, however. It is not to be inferred that these effects were the consequence of a deficiency in ACTH alone, since such animals evidently suffer from multiple hormonal deficiency. Parenthetically, it may be noted that the half life of ascorbic acid in guinea pigs is also very variable.⁷

TABLE 1

STEADY-STATE HALF LIFE OF ASCORBIC ACID IN IMMATURE SPRAGUE-DAWLEY RATS

Experiment	Weight (gm.)	Intact (hours)	Experiment	Weight (gm.)	Hypophysectomized (hours)
H-8	210	38	H-2	132	70
H-11	175	42	H-A	106	78
H-10	180	45	H-B	104	81
H-5	122	54	H-1	127	83
H-D	152	79	H-4	145	86
H-6	160	82	H-7	145	95
H-3	160	89	H-C	98	123
Mean		61			88

Data arrayed to show range of values. For difference between means, $P = 0.024$.

The mean turnover time is 88 hours for intact rats and 127 hours for hypophysectomized rats.

Depending upon the direction of concurrent changes, if any, in biosynthetic activity, the increase in half life of ascorbic acid in hypophysectomized rats may lead to an increase in the size of the body pool, or a decrease, or no change at all. Isolation of ascorbic acid from the entire carcass of the animal (a procedure that for several reasons appeared preferable to measurement of changes in specific activity of ascorbic acid in urine) left no doubt as to the fate of the body pool after hypophysectomy. Results are shown in TABLE 2. The specific activities of ascorbic acid isolated from control rats were substantially lower than those of the hypophysectomized rats, indicating that, in the latter, radioactive ascorbic acid had been incorporated into a smaller reservoir. In effect, this meant that the rate of synthesis had been diminished out of proportion to the rate of catabolism, so that the body pool contracted. Calculated values for the body pools in the two groups of rats are shown in TABLE 3. The highest value among hypophysectomized rats is that of a rat that had been placed in the metabolism chamber within two days of the operation. This may, in part, account for the relatively large body pool. However, since it is not possible to determine the body pool prior to the operation it is not possible to ascertain

the actual change. Such an animal could conceivably have experienced considerable diminution of the body pool and still fall in the upper range of operated rats, if it had an initially large body pool.

To confirm these measurements of total tissue ascorbic acid further, chemical analyses for ascorbic acid in various tissues and organs were performed, as

TABLE 2
SPECIFIC ACTIVITY OF TISSUE ASCORBIC ACID*

Experiment	Intact	Experiment	Hypophysectomized
H-8	3,200	H-A	14,200†
H-11	4,800	H-2	39,300
H-10	5,700	H-1	41,700
H-5	11,700	H-7	42,800
H-6	13,300	H-4	48,500
H-3	13,800	H-C	61,800
H-D	20,700	H-B	74,000

Data arrayed to show range. Time: 5 days after administration of ascorbic-1-C¹⁴ acid (1.0 mg., 3.75 μ c.)

* Specific activity given in cpm/mg.

† This animal had an anomalously high initial excretion of radioactivity (chiefly in respiratory CO₂), approximately 83 per cent of the dose in 24 hours. Its body pool (10.8 mg./100 gm. of body weight) and steady-state half life of ascorbic acid (78 hours) were within the range for its group. The low specific activity thus does not represent a true overlap between the two groups.

TABLE 3
TOTAL ASCORBIC ACID IN IMMATURE SPRAGUE-DAWLEY RATS*

Experiment	Intact	Experiment	Hypophysectomized
H-D	16.7	H-B	9.5
H-11	17.3	H-A	10.8
H-8	19.7	H-4	10.8
H-10	21.2	H-2	12.3
H-6	23.4	H-7	13.0
H-5	24.2	H-1	15.6
H-3	27.4	H-C	16.6
Mean	21.4		14.8

Data arrayed to show range.

The mean turnover rate is 0.24 mg. of ascorbic acid per hour per 100 gm. of body weight for intact rats, and 0.12 mg. per hour for hypophysectomized rats. Intact rats excrete approximately 40 per cent of the total synthesized into urine, but hypophysectomized rats only about 14 per cent, measured as total ascorbic acid.

* Ascorbic acid measured in mg./100 gm. body weight.

shown in TABLE 4. Because of the limited numbers of analyses involved, it is not intended to propose that these averages are truly representative values; however, they suffice to indicate the trend of events after hypophysectomy, and they confirm (by summation of the appropriate amounts of ascorbic acid contributed by each tissue) that measurements of body pools based on metabolism experiments cited above must be correct. Of particular consequence is the very marked decrease of ascorbic acid in muscle, bone, and skin. An

Additional reason why the body pool decreased after hypophysectomy is seen in TABLE 5, which indicates that some tissues, particularly visceral organs of high ascorbic acid concentration, tended to decrease in size after hypophysectomy. This was a contributing but evidently not a determining factor.

TABLE 4
ASCORBIC ACID IN TISSUES*

Tissue	Intact	Hypophysectomized	
		3-8 days	7 weeks
Heart	14.6	15.2	16.5
Liver	40.2	29.1	32.6
Lungs	45.7	31.1	33.0
Testes	48.5	56.0	46.6
Kidneys	18.7	20.2	18.8
Spleen	53.3	60.1	60.8
Small intestine	51.0	37.4	41.1
Adrenals	576	372	324
Brain	42.1	44.9	51.8
Bone	12.9	9.7	5.8
Muscle	8.2	5.7	5.3
Skin	21.5	15.8	10.0

* Values represent averages of 3 to 4 individual measurements (mg./100 gm. tissue weight)

TABLE 5
ORGAN WEIGHTS*

Tissue	Intact	Hypophysectomized		
		3-8 days	6 weeks	7 weeks
Heart	0.35	0.34	—	0.36
Liver	4.44	4.53	2.99	3.55
Lungs	0.98	0.64	0.57	0.72
Testes	1.38	0.69	0.32	0.15
Kidneys	0.90	0.76	0.56	0.73
Spleen	0.32	0.27	0.23	0.14
Small intestine	2.81	3.30	2.40	—
Adrenals	0.023	0.015	0.007	0.012
Brain	1.35	1.65	—	1.78
Femur	0.36	0.35	—	0.41
Skin	15.8	15.6	18.8	15.17
Thymus	0.20	0.22	0.32	—

* Values represent averages of two individual measurements, except those of intact rats, which are based on three (gm./100 gm. body weight).

In view of the decrease in rate of biosynthesis after hypophysectomy, it was anticipated that urinary excretion of ascorbic acid should also be diminished. The surprising aspect was the precipitous decline after the operation, shown in FIGURE 2. Within 48 hours a new low level of excretion was established, which was maintained unchanged for well over one month, or as long as measurements were continued (see FIGURE 3). Hypophysectomy thus caused an immediate and, without treatment, irreparable defect, indicating

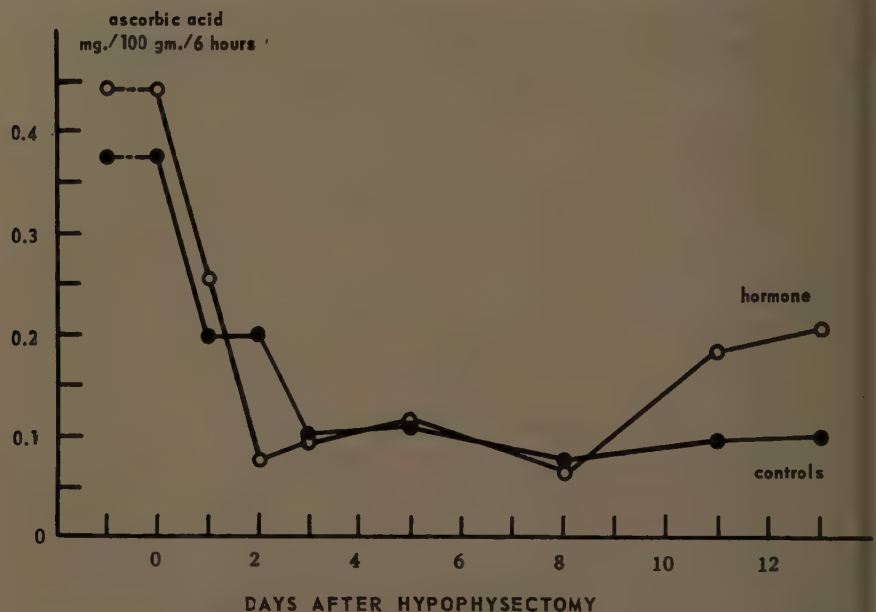


FIGURE 2. The precipitous decrease in excretion of urinary ascorbic acid after hypophysectomy is complete within 2 days after the operation. Growth hormone was administered starting from the day of the operation, but its effect was not apparent until more than 1 week later. Initial weight of all rats, approximately 100 gm. Urine samples were collected for 6 hours in these experiments during the morning and early afternoon in iced collection flasks.

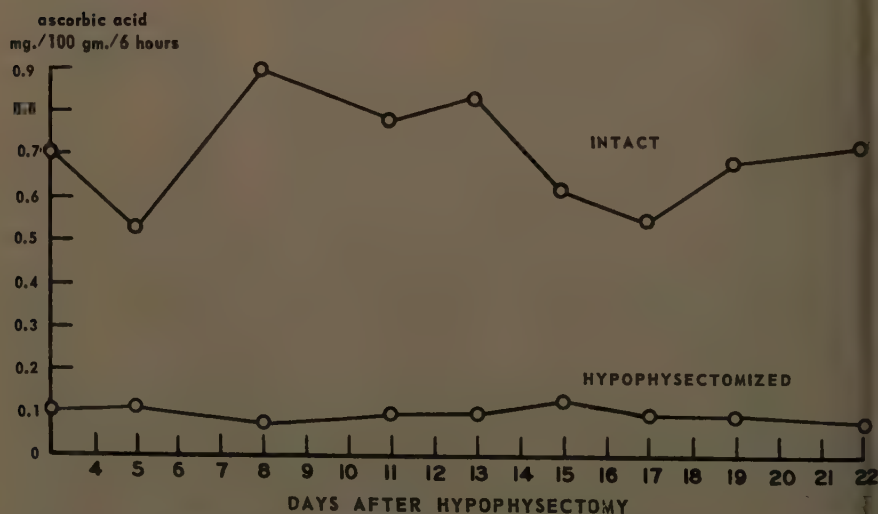


FIGURE 3. Urinary excretion of ascorbic acid by intact rats and by hypophysectomized rats without further treatment. Actual measurements extended over 1 month.

very marked dependence of some reaction or sequence of reactions upon availability of pituitary hormone.

In an effort to discover the nature of the hormonal deficiency, a task not very promising because of the possible simultaneous involvement of a multiplicity of hormones, the half life and body pool of ascorbic acid in thyroidectomized rats was determined. Metabolism experiments were started about 5 days after removal of the thyroids, which is not long enough to cause severe consequences of thyroid insufficiency. It is to be recalled, however, that effects on ascorbic acid metabolism were very much in evidence at such a time in hypophysectomized rats. It appeared that neither the body pool nor the half life of ascorbic acid was in any detectable way affected by thyroidectomy, as shown in TABLE 6. Similarly, neither ACTH in doses of 4 I.U. of gel* daily by subcutaneous injection nor cortisone acetate in daily subcutaneous doses of 5 mg. was able to increase urinary excretion of ascorbic acid and presumably had no effect on the body pool.

Growth hormone in relatively small doses finally did produce a response in terms of urinary excretion of ascorbic acid. Administered in daily intraperi-

TABLE 6
ASCORBIC ACID IN THYROPARATHYROIDECTOMIZED RATS

Experiment	Weight (gm.)	$t_{1/2}$ (hours)	Total pool (mg. %)	Specific activity* (corr., cpm/mg.)
T-1	258	83	23.1	12,100
T-2	275	41	18.8	7,400

* Duration of metabolism experiments 5 days. Specific activity corrected to a dose of 0.0 mg. (3.75 μ c.) of ascorbic-1- C^{14} acid.

oneal amounts of 150 γ , which produced less than half-maximal growth in these hypophysectomized rats, it caused increased excretion of reduced (FIGURE 4) and total ascorbic acid (FIGURE 5) in separate experiments over the entire experimental period, extending considerably beyond that shown in FIGURE 5 to more than 1 month. When hormone was withdrawn, excretion fell to the level of untreated rats. When hormone was administered to the control group, excretion of ascorbic acid soon increased. It is likely that higher doses of hormone would have produced a more marked response, but they were avoided because undesirable results of chronic administration might have introduced complications under those circumstances. The effect of growth hormone on the half life of ascorbic acid in hypophysectomized rats is shown in TABLE 7. The half life appears to be diminished relative to that in operated rats without treatment, that is, it appears to be more nearly like that of intact rats.

In remarkable contrast to the effect on ascorbic acid, the excretion of total glucuronic acid (the sum of free and conjugated glucuronic acid) was not nearly as much affected by hypophysectomy, nor was there any significant difference between the groups of hypophysectomized rats as a result of administration of growth hormone (FIGURE 6). Furthermore, even the differences between

* Organon, Inc., Orange, N.J.

intact and hypophysectomized rats disappeared during the course of the experimental period because of the gradual reduction in excretion of glucuronic acid, which was more marked in intact than in operated rats. After about 30 days, urinary total glucuronic acid per 100 gm. of body weight in all groups was in effect identical.

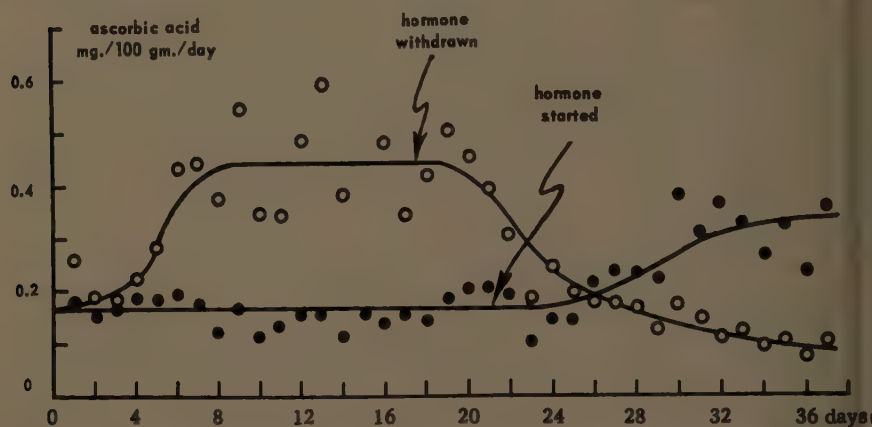


FIGURE 4. Urinary excretion of reduced ascorbic acid by hypophysectomized rats. Each point represents the average of three values measured by titration with 2,6-dichlorobenzene-indophenol. Initial weights of all rats, approximately 100 gm. Growth hormone was administered to the test group (open circles) in doses of 150 μ g. per day until arrow indicates withdrawal. Hormone was then administered to the original control group. Twenty-four-hour collection periods.

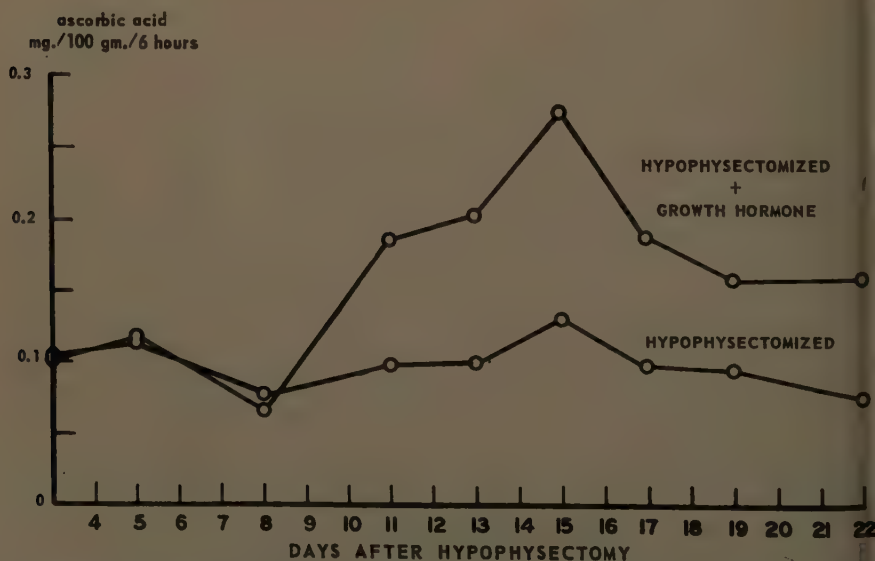


FIGURE 5. Urinary excretion of ascorbic acid by hypophysectomized rats with or without growth hormone. Six-hour collection periods.

This information is not sufficient to delineate either the nature of the defect in hypophysectomized rats or the action of growth hormone on this system. The observations are, however, consistent with the view that there is in both instances a more specific effect on reactions lying between glucuronic acid and

TABLE 7
STEADY-STATE HALF LIFE OF ASCORBIC ACID IN HYPOPHYSECTOMIZED RATS
AFTER TREATMENT WITH GROWTH HORMONE*

Experiment	Weight (gm.)	Half life (hours)
S-A	169	63
S-B	169	53
S-C	177	64
S-D	155	79
Mean		65

* 150 γ intraperitoneally per day for 30 (S-A, S-B) and 35 days (S-C, S-D).

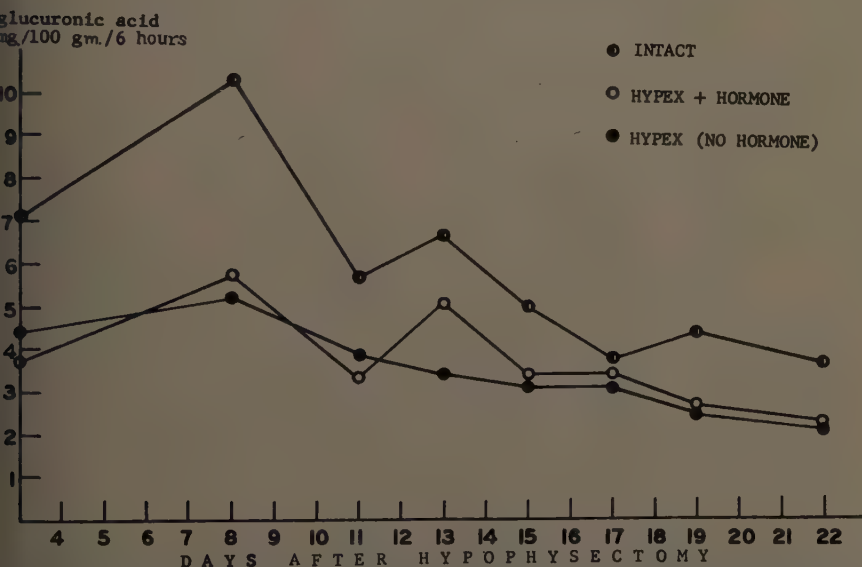


FIGURE 6. Urinary excretion of glucuronic acid in intact rats and hypophysectomized rats with or without growth hormone. The gradually diminishing differences ultimately disappeared entirely. Six-hour collection periods.

ascorbic acid than on the production of glucuronic acid or the uronate pathway⁸ in general.*

Much the same conclusion may be obtained by examination of data from

* More recent findings support this view. Depressed activities of aldolactonase primarily and L-gulonolactone oxidase secondarily alone are adequate to explain the defect in synthesis of ascorbic acid. Uronolactonase activity, for example, is not affected by hypophysectomy.

experiments in which groups of the three categories of rats (intact, hypophysectomized, and hypophysectomized-growth hormone-treated) received doses of Chloretone by stomach tube. The data shown in FIGURE 7 were obtained on the fourth day after determination of the base level of urinary excretion of ascorbic and glucuronic acids, and the start of administration of 9 mg. of Chloretone per 100 gm. of body weight. The hormone-treated group received, in addition, its regular injections before and during the entire period. During the initial 6 hours after administration of the fourth dose of Chloretone, there was found to occur sharply increased excretion of ascorbic acid in all groups:

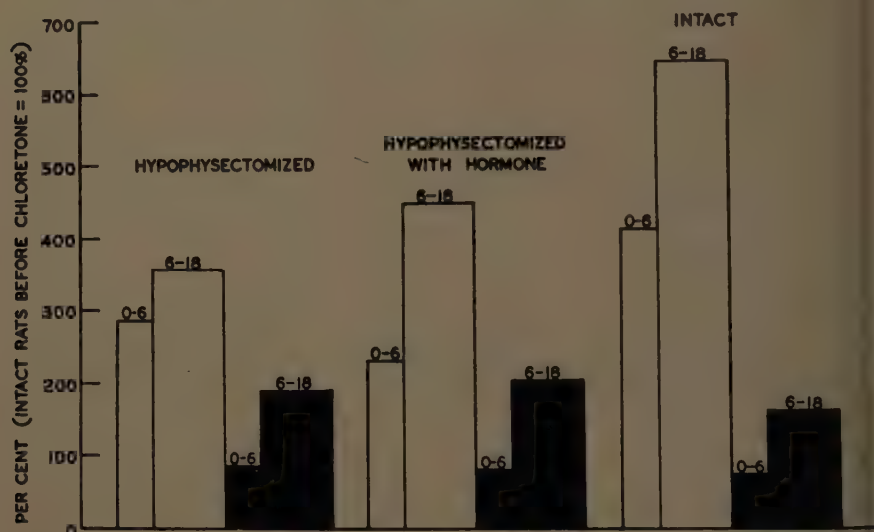


FIGURE 7. Urinary excretion of ascorbic acid (*open bars*) and glucuronic acid (*solid bars*) on the fourth day of administration of Chloretone. Numbers over bars indicate hours after chloretone administration. The levels of glucuronic acid may be seen to be essentially unchanged from the basal level during the initial 6 hours, while ascorbic acid has already increased substantially. Values are calculated for excretion per 100 gm. of body weight, and are expressed as per cent of the excretion of ascorbic acid or glucuronic acid, respectively, by the intact group of rats prior to treatment with Chloretone. Four animals in each group, except that glucuronic acid of intact rats is based on two animals.

although an earlier report indicated that hypophysectomized rats should be unresponsive.⁹ A further substantial increase in excretion of ascorbic acid occurred during the subsequent 12 hours. It is of considerable interest to note that the results summarized in FIGURE 7 show a persistent effect of growth hormone even after Chloretone stimulation, and higher excretion of ascorbic acid by intact chloretone-treated rats than by those who were operated. However prior to administration of the drug, the ratio of excretion in intact, hypophysectomized-hormone-treated, and hypophysectomized controls was 100:34:18 (0.56:0.19:0.10 mg. per 6 hours), but it was 100:69:56 (3.6:2.5:2.0 mg. per 6 hours) during the peak excretion period. Relative differences became drastically smaller. Thus the unphysiological stimulus provided by Chloretone evoked a degree of activity that was not greatly different in rats with an ap

parently defective synthetic mechanism than in those presumably normal in this respect.

Urinary excretion of glucuronic acid did not differ greatly among the three groups of rats before administration of Chloretone. Furthermore, they responded to approximately equal extents to the drug, so that there was no appreciable difference afterward. The relative increase in excretion of ascorbic acid was found to be much greater than that of glucuronic acid, especially, of course, in the hypophysectomized groups. Thus in respect to excretion of ascorbic acid and glucuronic acid, there was not a considerable difference between chloretonized intact and hypophysectomized rats, although differences without the drug were marked. The conclusion that may be reached from these data is that the effect on synthesis of ascorbic acid by Chloretone goes beyond that on glucuronic acid, much as was the case with growth hormone. Provided that urinary excretion of these substances is an adequate indicator of synthetic activity, it would appear that the action of growth hormone and Chloretone on one of the enzymic reactions between glucuronic acid and ascorbic acid transcends that which they may have on any of the related reactions

TABLE 8
REDUCED PYRIDINE NUCLEOTIDES IN LIVERS OF RATS*

	DPNH	TPNH
Intact	0.298 0.270	0.324 0.371
Hypophysectomized	0.272 0.305	0.462 0.595

* Measured in μ mole/gm.

under discussion; that urinary excretion of the two compounds may be, or may become, relatively independent; and that increased excretion of ascorbic acid is not necessarily the sole consequence of increased synthesis of glucuronic acid. One alternate possibility is the existence of another route to ascorbic acid that does not involve glucuronic acid. This hypothesis does not seem amenable in consideration of published evidence on the biosynthesis of ascorbic acid.

If the TPN-L-hexonate dehydrogenase reaction is inhibited in hypophysectomized rats, it is unlikely that a deficiency of TPNH (or lowering of activity of pathways furnishing TPNH, such as the hexose monophosphate shunt) is responsible. Measurement of concentrations of DPNH and TPNH¹⁰ in livers of hypophysectomized and intact rats disclosed that the concentration of DPNH was not altered by removal of the pituitary but that TPNH was actually *increased* by a very substantial amount (see TABLE 8). The significance of this is now under further study.

Summary

It has been found that ACTH depresses the catabolism of ascorbic acid as judged by decrease in respiratory radioactivity originating from ascorbic-1-C¹⁴

acid. In hypophysectomized rats the half life of ascorbic acid is increased (and turnover rate decreased), the body pool is decreased, and urinary excretion falls off precipitously after removal of the pituitary. Neither thyrotropic hormone, ACTH, nor cortisone appears to affect this course of events significantly, but growth hormone stimulates urinary excretion of ascorbic acid and diminishes the half life.

Urinary excretion of glucuronic acid is less severely depressed after hypophysectomy and gradually becomes equal to that of intact rats. Growth hormone has no apparent effect on urinary excretion of glucuronic acid. These findings indicate that the defect in synthesis of ascorbic acid after hypophysectomy resides more specifically, but not necessarily exclusively, in a step between glucuronic acid and ascorbic acid and that the partial reversal by suboptimal doses of growth hormone is similarly relatively specific.

Hypophysectomized rats, with or without pretreatment with growth hormone, respond well to administration of Chloretone by increased urinary excretion of ascorbic acid. Per unit of body weight, levels found are not greatly lower than those excreted by intact rats in spite of the pronounced differences prior to stimulation by Chloretone. The effect of Chloretone on excretion of glucuronic acid is relatively much less in all groups of rats, and there is little difference between them either before or during treatment with the drug. This is interpreted to indicate that Chloretone stimulates accelerated excretion of ascorbic acid not so much because of a mass action effect, resulting from increased production of glucuronic acid, but because of a more specifically directed action on an enzymic step between glucuronic acid and ascorbic acid.

In intact rats, the steady-state half life of ascorbic acid and turnover time are, respectively, 61 and 88 hours, the body pool 21 mg., and the turnover rate about 0.24 mg. per hour, per 100 gm. of body weight; roughly 40 per cent of the amount synthesized is excreted as total ascorbic acid in urine. In hypophysectomized rats, the half life is 88 hours, the turnover time 127 hours, the body pool is 15 mg., and the turnover rate 0.12 mg. per hour, per 100 gm. of body weight; about 14 per cent of synthesized material is excreted as total ascorbic acid.

The concentration of DPNH appears to be the same in livers of intact and hypophysectomized rats, but that of TPNH is substantially increased by hypophysectomy.

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STUDIES ON THE TISSUE DISTRIBUTION OF ASCORBIC ACID

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While it is well known that the concentration of ascorbic acid varies markedly in different tissues, the factors regulating its distribution are not understood. In the present study an attempt has been made to investigate the mechanism whereby ascorbic acid is taken up by various tissues from the circulating blood.

Distribution of L-Ascorbic Acid-1-C¹⁴

Small doses of L-ascorbic acid-1-C¹⁴ were injected into the hearts of rats. The animals were sacrificed at various times, and the amount of L-ascorbic acid-1-C¹⁴ in the various tissues was determined. The data presented in FIGURE 1 indicate that L-ascorbic acid-1-C¹⁴ is rapidly removed from the serum and transferred to such tissues as adrenal, kidney, and liver. In the early time periods, however, such tissues as eye, muscle, testis, and brain accumulate only relatively small amounts of radioactive ascorbic acid. As shown in FIGURE 2, the specific activity of ascorbic acid in adrenal, kidney, and liver rapidly equaled that found in the serum, and these tissues remained in equilibrium throughout the period investigated. The specific activities of ascorbic acid in eye, testis, muscle, and brain were markedly different from serum, and these tissues only slowly equilibrated with serum. It is also evident from FIGURES 1 and 2 that the rate at which a tissue accumulates radioactive ascorbic acid and equilibrates with serum ascorbic acid is in general unrelated to the amount of ascorbic acid present in the tissue.

Form of the Vitamin Transported

In order to explain these results, attention was directed to the form of ascorbic acid required for its penetration into cells. Ascorbic acid can exist in two readily interconvertible forms (FIGURE 3). The reduced form of the vitamin is a relatively strong acid (pK_a 4.17), whereas the oxidized form of the vitamin (dehydroascorbic acid) is nonionic and more lipoid-soluble.† For this reason, dehydroascorbic acid would be expected to cross cellular barriers more readily than ascorbic acid. This difference in properties has been previously recognized, and several investigators have suggested dehydroascorbic acid as the form of the vitamin penetrating erythrocytes,⁴⁻⁶ the placental barrier,⁷ and brain.⁸

In order to evaluate the relative rates at which ascorbic acid and dehydroascorbic acid penetrate cell barriers, small doses of ascorbic acid-1-C¹⁴ or dehydroascorbic acid-1-C¹⁴ were administered to rats, and the specific activity was determined in serum, eye, and brain (FIGURE 4). It is evident that the ascorbic acid in brain and eye approached equilibrium with serum more rapidly when the oxidized form of the vitamin was administered.

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† The distribution coefficients between *n*-butanol and water at pH 7.4 are 0.01 for ascorbic acid and 0.10 for dehydroascorbic acid.

A similar comparison of the relative ability of ascorbic acid and dehydroascorbic acid to penetrate cellular barriers was measured in red blood cells *in vitro* (TABLE 1). Red blood cells incubated with radioactive dehydroascorbic

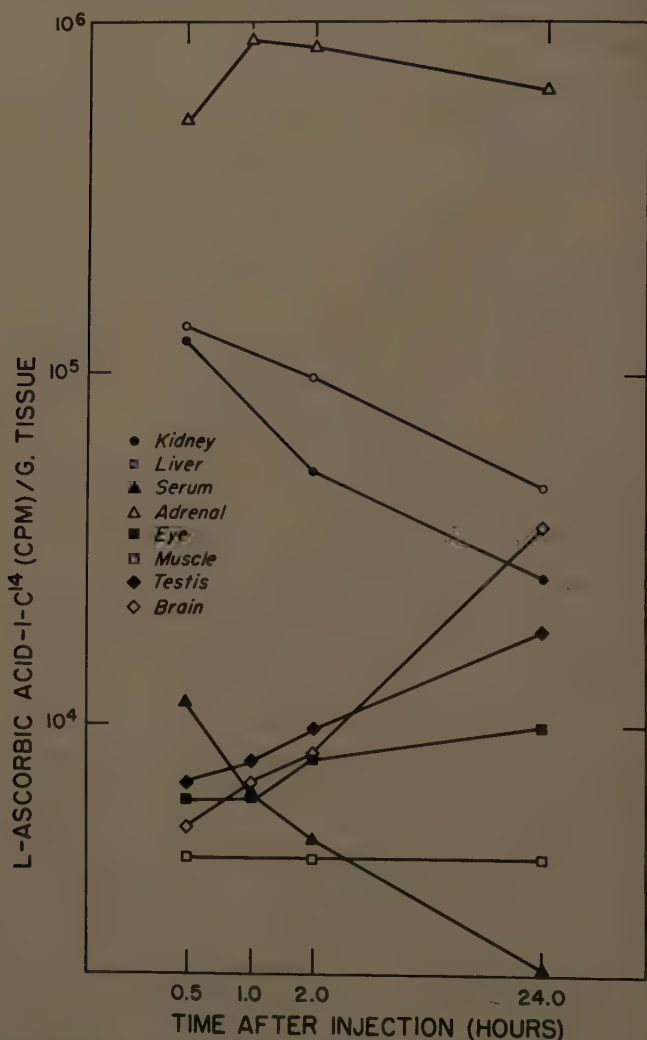


FIGURE 1. Tissue levels of L-ascorbic acid-1-C¹⁴ at various times after I.V. administration. The C¹⁴ present in various tissues as L-ascorbic acid was determined by a carrier dilution method.¹ Ascorbic acid was determined by the method of Roe *et al.*^{2,3}

acid were found to accumulate considerably more of the vitamin than red blood cells incubated with radioactive ascorbic acid.

Further evidence indicating that dehydroascorbic acid is the form preferred for transport across cellular barriers is presented in TABLE 2. Twenty mg. of dehydroascorbic acid or ascorbic acid was injected into the hearts of rats.

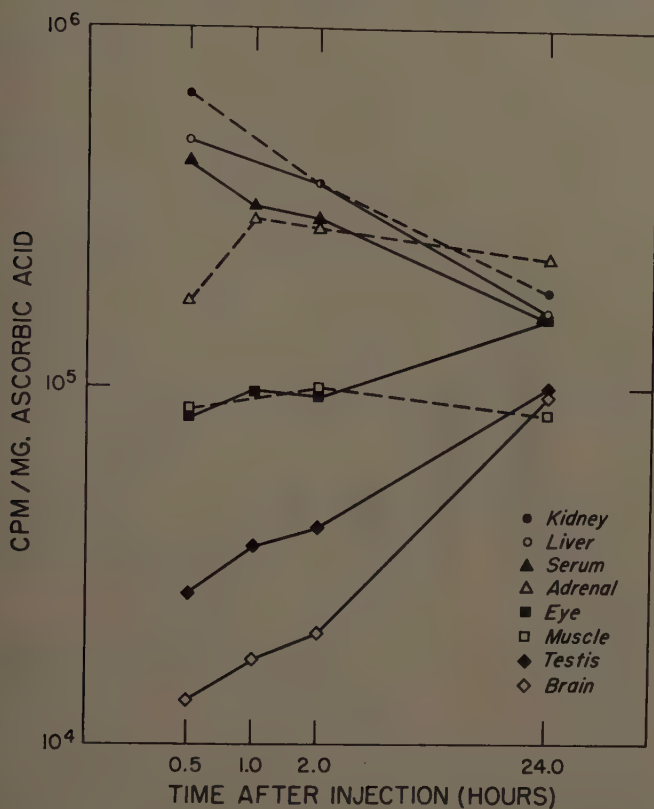


FIGURE 2. Specific activity of ascorbic acid in various tissues following the injection of ascorbic acid-1-C¹⁴.

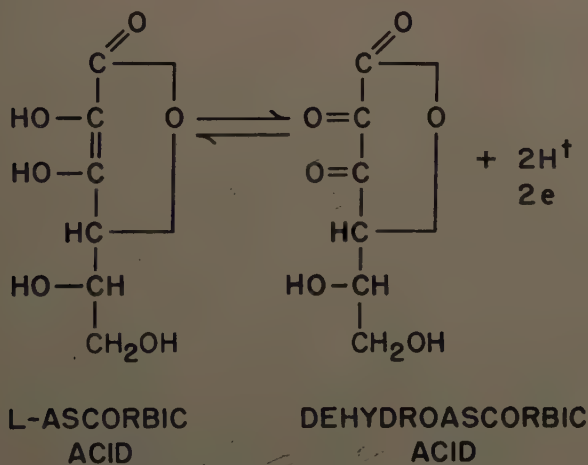


FIGURE 3. Interconvertible forms of ascorbic acid.

These animals were sacrificed after injection, and the levels of ascorbic acid in the brain were measured. The administration of 20 mg. of dehydroascorbic acid produced a rapid and prolonged elevation in ascorbic acid levels in this tissue. The brain levels of dehydroascorbic acid plus diketogulonic acid were measured in these animals and found to be essentially the same as those found in control rats (approximately 2 mg. per cent). The administration of 20 mg

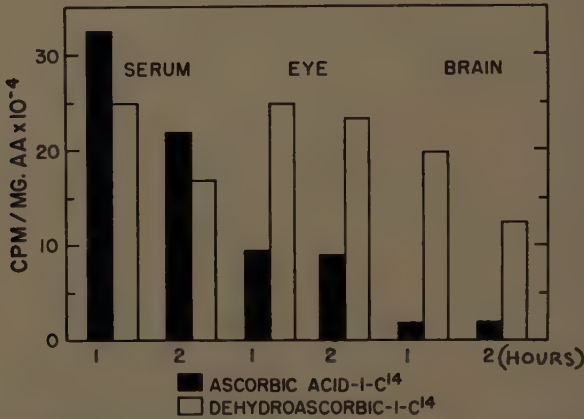


FIGURE 4. Specific activity of ascorbic acid in various tissues following the injection of L-ascorbic acid-1-C¹⁴ or dehydroascorbic acid-1-C¹⁴.

TABLE 1
PENETRATION OF L-ASCORBIC ACID-1-C¹⁴ (L-AA-1-C¹⁴) OR DEHYDROASCORBIC ACID-1-C¹⁴ (DHA-1-C¹⁴) INTO RAT ERYTHROCYTES *IN VITRO**

Substance incubated	Ascorbic acid (cpm/μg.)	
	Cells	Supernate
L-AA-1-C ¹⁴	50	2000
L-AA-1-C ¹⁴	250	1900
DHA-1-C ¹⁴	800	2000
DHA-1-C ¹⁴	610	1800

* Whole blood was collected from rats in heparin, 1 ml. diluted to 4 ml. with Krebs-Ringer phosphate, pH 7.4. Twenty μg. of L-AA-1-C¹⁴ or DHA-1-C¹⁴ (2000 cpm/μg.) was added to each flask and incubated at 37° C. for 30 min. Cells were removed from supernate by centrifugation and washed twice with 10 ml. Krebs-Ringer phosphate, pH 7.4.

of ascorbic acid did not change the level of ascorbic acid in the brain. These results indicate that dehydroascorbic acid readily penetrates into the brain and is then reduced to ascorbic acid whereas under these same conditions ascorbic acid penetrates slowly if at all.

D-Isoascorbic acid, D-ascorbic acid, D-glucoascorbic acid, and their respective dehydro forms were also administered to rats in 20- and 40-mg. dosages. The administration of these compounds did not increase the level of ascorbic acid like material in the brain. Since these compounds have essentially the same distribution coefficients between butanol and water as previously stated for

ascorbic acid and dehydroascorbic acid, this finding suggests that the transport or storage mechanism in brain for ascorbic acid is structurally specific. The inability of these analogues to cross cellular barriers may be an important factor in explaining their low antiscorbutic activity.

Role of the Kidney

One possible explanation of the distribution pattern of radioactive ascorbic acid is that it is oxidized to dehydroascorbic acid prior to penetration into

TABLE 2
BRAIN LEVEL OF L-ASCORBIC ACID AFTER I.V. INJECTION OF 20 MG.
OF L-ASCORBIC ACID OR DEHYDROASCORBIC ACID

Time (minutes)	L-Ascorbic acid (mg.%)	Dehydroascorbic acid (mg.%)
0	49 \pm 1 (3)*	54 \pm 1.0 (9)
2		89, 79
5	55 \pm 2 (3)	86 \pm 3 (8)
15	54 \pm 0.3 (3)	77 \pm 7 (5)
30	50 \pm 0.6 (3)	82 \pm 7 (5)
60	51 \pm 2 (3)	76 \pm 5 (4)

* Values reported are the mean followed by the standard error. Numbers in parentheses indicate number of animals.

TABLE 3
RELATIVE SPECIFIC ACTIVITY* OF ASCORBIC ACID IN VARIOUS TISSUES 2
HOURS AFTER L-ASCORBIC ACID-1-C¹⁴ ADMINISTRATION

Tissue	Intact rats†	Nephrectomized rats†
Serum	1.0	1.0
Liver	1.6	0.55
Adrenal	1.32	0.44
Eye	0.40	0.10
Testis	0.21	0.11
Brain	0.09	0.07

* Specific activity of ascorbic acid in serum taken as 1.0.

† Average value found in 3 animals.

issues but that only certain tissues are able to oxidize extracellular ascorbic acid. Since the acidity of tubule urine would be expected to facilitate the diffusion of ascorbic acid into kidney cells where its oxidation to dehydroascorbic acid might occur, the role of the kidney in the distribution of the vitamin is investigated.

L-Ascorbic acid-1-C¹⁴ was injected into the hearts of intact and nephrectomized animals, and the specific activity of ascorbic acid was measured in various tissues. The data in TABLE 3 indicate that nephrectomy causes a marked lag in the accumulation of ascorbic acid by various tissues.

In order to study further the role of the kidney in the distribution of ascorbic acid, investigations were carried out in rats treated with Chloretone (chlorotantalol), a compound that increases the rate of ascorbic acid biosynthesis.^{9,10}

The administration of 20 mg. of Chloretone for 3 days to intact rats resulted in a moderately elevated level of ascorbic acid in serum and a marked increase in liver and muscle (FIGURE 5). Rats that had been treated with Chloretone for 2 days were nephrectomized and sacrificed 24 hours later. Nephrectomizing the animals treated with Chloretone resulted in an enormous increase in serum ascorbic acid without much effect on the level of ascorbic acid in liver or muscle. This suggested that ascorbic acid was not accumulating in the

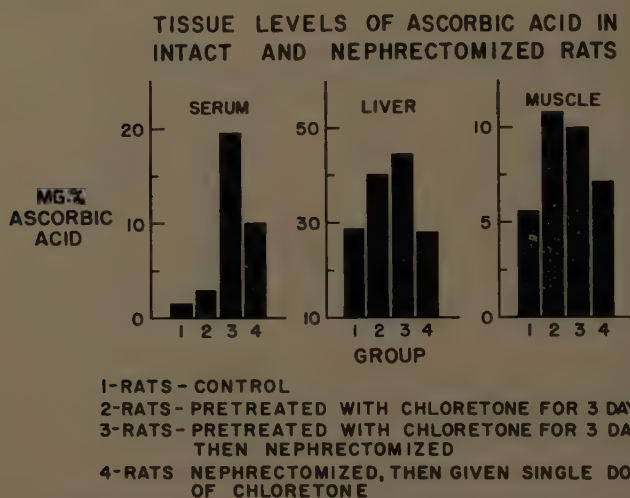


FIGURE 5. Effect of accelerated biosynthesis of ascorbic acid on tissue levels in intact and nephrectomized rats.

TABLE 4
FORM OF VITAMIN C IN SERUM FROM VARIOUS BLOOD VESSELS

Serum source*	Total (mg.%)	Oxidized (mg.%)	Per cent of total oxidized
Carotid artery	1.45	0.62	43
Jugular vein	1.35	0.52	39
Femoral vein	1.43	0.70	49
Renal vein	1.33	1.03	77
Mesenteric vein	1.19	1.04	87

* Average of 2 dogs.

tissues of nephrectomized rats. In order to demonstrate that the failure to accumulate ascorbic acid in the tissues was not due to tissue saturation, further experiments were carried out in rats that were first nephrectomized and then treated with 20 mg. of Chloretone. Twenty-four hours later the animals were sacrificed and tissue levels of ascorbic acid were determined. Again there was a marked increase in the serum level of ascorbic acid while tissue levels remained at control levels. These and the previous findings suggest that there is a defect in the transport of ascorbic acid from serum to tissues in nephrectomized animals.

The kidney has been demonstrated to have a potent ability to oxidize ascorbic acid *in vitro*.¹¹ Evidence indicating that the kidney also oxidizes ascorbic acid *in vivo* is presented in TABLE 4. Blood was drawn from various blood vessels of dogs and analyzed for reduced and oxidized forms of ascorbic acid. Serum obtained from the carotid artery and from the jugular and femoral veins was found to contain less oxidized ascorbic acid than did serum obtained from the renal vein. Serum obtained from the renal and mesenteric veins contained ascorbic acid largely in the oxidized form. This finding is consistent with the theory that the kidney and possibly the intestine facilitate the transport of ascorbic acid by oxidizing the vitamin to an un-ionized form that readily penetrates cellular barriers.

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CATABOLISM OF L-ASCORBIC-1-C¹⁴ ACID AS A MEASURE OF ITS UTILIZATION IN THE INTACT AND WOUNDED GUINEA PIG ON SCORBUTIC, MAINTENANCE, AND SATURATION DIETS

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It has been known for nearly two centuries that scurvy could be prevented and cured by a substance contained in citrus fruits and fresh vegetables. It is known that a scorbutic individual resumes normal anatomic structure and metabolism solely by the repletion of ascorbic acid. Whether the depleted tissues need to metabolize vitamin C rapidly or whether they need to accumulate and store ascorbic acid in order to recover normal function is a problem that has not been clarified.

Another intriguing problem is the part played by ascorbic acid in surgical wound healing. While the vitamin is known to be essential for this process, the same question arises as with intact tissues: Is the ascorbic acid metabolized more rapidly or is it needed for storage and retention?

We believe that the study of the catabolism of L-ascorbic-1-C¹⁴ acid administered in a single injection has given us the answers to these questions.

The experiments of Penney and Zilva¹ demonstrated that various modes of administration of ascorbic acid resulted in differences in deposit in tissues. Therefore, we chose three diets of vitamin C, from depletion to saturation, to determine accurately repletion and the mechanism involved in tissue wound healing. The methods and materials have been described in the main in previous publications.²⁻⁶

We have modified and simplified the metabolic chamber illustrated in FIGURE 1. An inverted dessicator served as the chamber, and contained a perforated can filled with calcium chloride to absorb excess moisture. A rotary air-blast and-suction apparatus was used. A flow meter consisting of an absorption tower, 15 in. in over-all height, was filled with concentrated sulfuric acid (FIGURE 2). An inner glass dome with inner seal will lift to the desired level according to the flow of air through the system. This proved superior to a manometer facilitating air flow adjustment.

We³ have shown that, following injection of radioactive ascorbic acid, the scorbutic animal will retain a greater proportion of the injected dose than an animal on a maintenance diet of ascorbic acid 6 mg. daily (TABLE 1).

Curves for cumulative C¹⁴O₂ excretion (FIGURE 3) for three groups of guinea pigs on different dietary intakes demonstrated a qualitative similarity but a quantitative difference. Immediately following administration of the labeled ascorbic acid, a high rate of C¹⁴O₂ expiration occurred, leveling out after approximately 48 hours. The animals on the highest intake showed the greatest excretion of C¹⁴O₂, while those at scurvy level showed the least excretion.

In addition to comparing the C¹⁴O₂ respiratory excretion to the dietary intake, we have correlated this excretion to the blood levels of individual guinea pigs (FIGURE 4). There is an excellent direct relationship.



FIGURE 1. Metabolic train for use in collection of respiratory carbon dioxide and urine. Modified after Mackenzie *et al.*,⁷ by Jackel *et al.*,⁸ and von Schuching *et al.*,⁵ The flow meter is located at the inlet and features an absorption tower with inner glass dome that can be adjusted easily to give the desired air flow. Reproduced by permission of the *American Journal of Physiology*.⁵

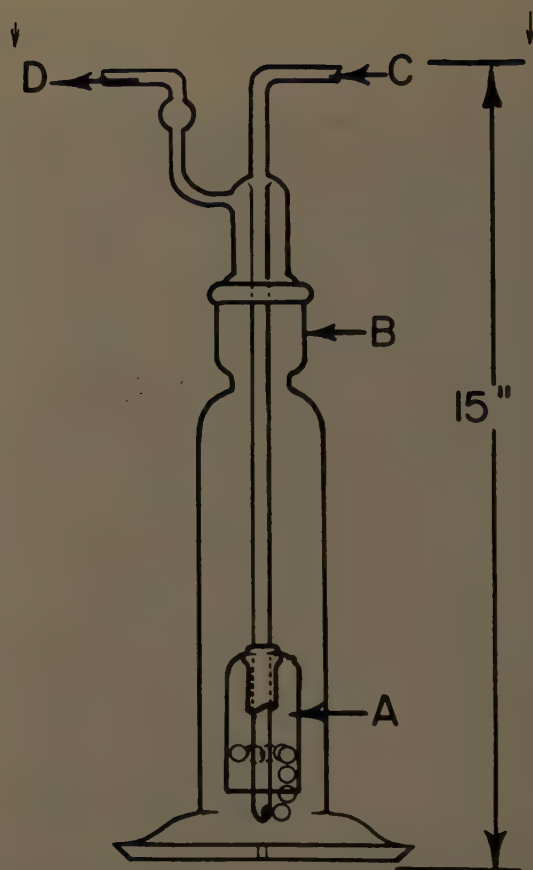


FIGURE 2. Flow meter used for regulating the air inlet through the metabolic train. The flow meter consists of an absorption tower, 15 inches over-all height. It is filled with concentrated sulfuric acid and placed in line directly before the metabolic chamber. The inner dome (A) will lift to the desired level, which can be marked on the outside of the tower; (B) standard taper 29/42 F; (C) air inlet; and (D) air outlet. Reproduced by permission of the *American Journal of Physiology*.⁵

TABLE 1
PERCENTAGE OF L-ASCORBIC-1-C¹⁴ ACID RETAINED PER GRAM OF TISSUE FROM
GUINEA PIGS 24 HOURS AFTER INJECTION ON SCORBUTIC AND
MAINTENANCE DIETS*

Tissue	Daily ascorbic acid intake†	
	0 mg.	6 mg.
Bone	0.44	0.18
Liver	0.60	0.20
Tendon	0.06	0.02
Blood	0.08	0.04
Scar muscle	0.30	0.10
Distant muscle	0.12	0.06

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† Data taken from averages of tissues from 2 or more guinea pigs.

DAILY ASCORBIC ACID INTAKE

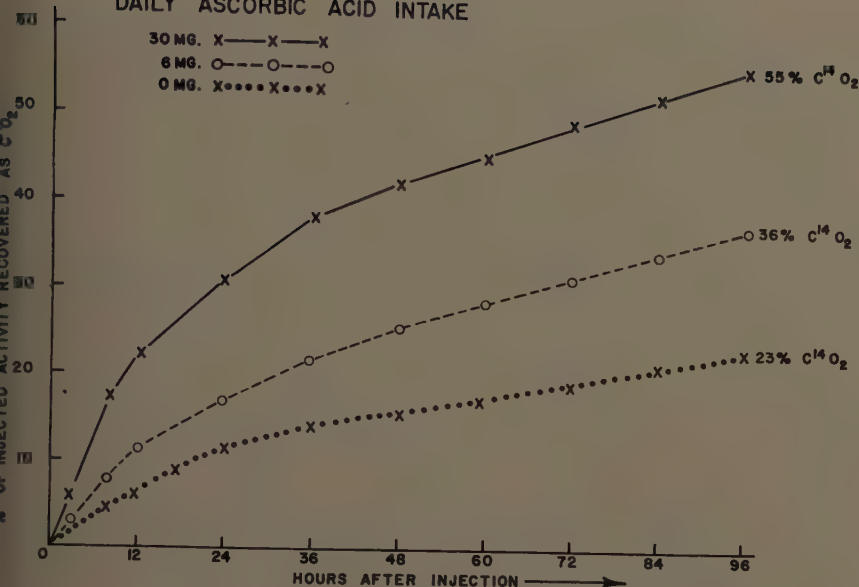


FIGURE 3. Carbon¹⁴ dioxide in exhaled air plotted for a 96-hour period as percentage of mg. L-ascorbic-1-C¹⁴ acid activity injected intramuscularly into the intact guinea pig. Three groups on varying dietary intakes of ascorbic acid are shown. Each curve represents average of data of 2 or more animals. Reproduced by permission of the *American Journal of Physiology*.⁵

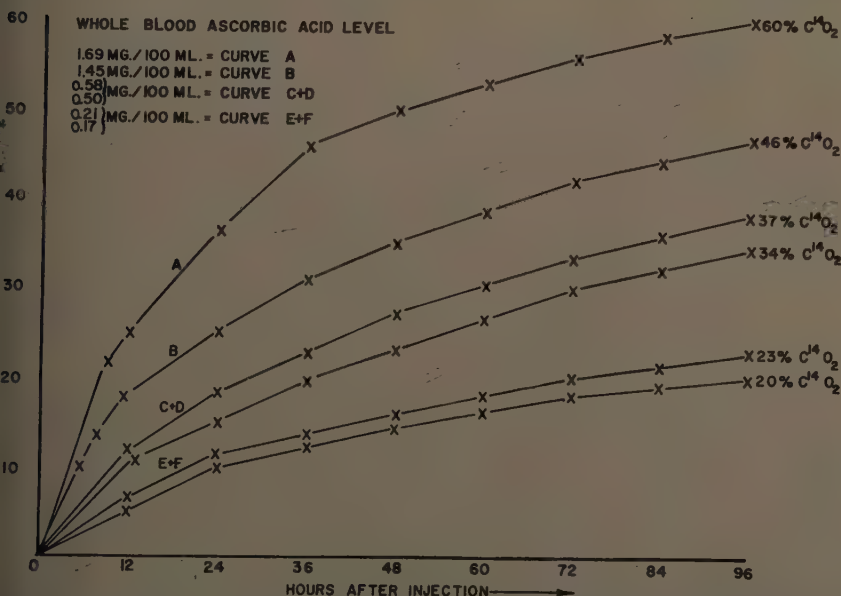


FIGURE 4. Carbon¹⁴ dioxide in exhaled air following single injection intramuscularly of mg. L-ascorbic-1-C¹⁴ acid. A comparison of curves from individual animals with varying whole blood ascorbic acid levels. Reproduced by permission of the *American Journal of Physiology*.⁵

Radioactivity excreted in the urine, in contrast to the excretion of radioactive CO_2 in the expired air, showed slightly higher values in the scorbutic groups. This is small but consistent (TABLE 2).

The retention of radioactivity 4 days following a single injection of C^{14} -tagged ascorbic acid is greatest in the scorbutic, and lowest in the saturated

TABLE 2
ACTIVITY OF C^{14} IN URINE AS PERCENTAGE OF 2-MG. L-ASCORBIC-1- C^{14} ACID INJECTED INTRAMUSCULARLY*

Hours	Daily ascorbic acid intake†					
	0 mg.		6 mg.		30 mg.	
	Before operation	After operation	Before operation	After operation	Before operation	After operation
48	14	15	13	14	10	14
96	23	—	19	18	11	18

* Reproduced by permission of the *American Journal of Physiology*.⁵

† Data represent averages from 2 or more guinea pigs.

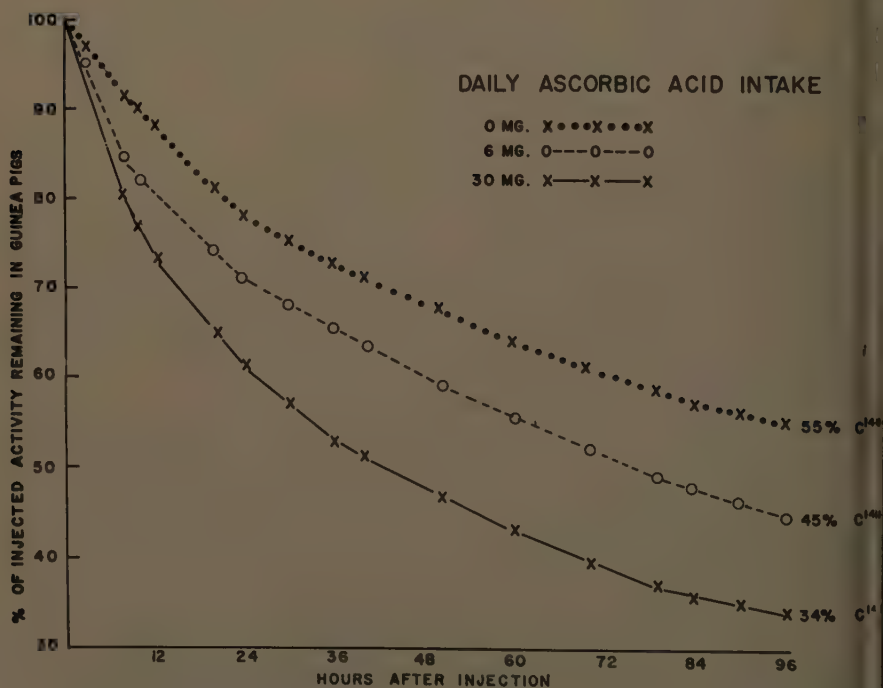


FIGURE 5. Carbon 14 radioactivity retained by guinea pigs expressed as percentages of intramuscularly injected 2-mg. L-ascorbic-1- C^{14} acid. Curves from animals on three levels of ascorbic acid intake are shown. The data are averages obtained from 2 or more guinea pigs. Reproduced by permission of the *American Journal of Physiology*.⁵

animal (FIGURE 5). This finding is made by subtracting the combined percentile activity in the expired air and urine from 100.

We have found the retained activity in muscle, tendon, liver, adrenal, and periosteum to consist of ascorbic acid, dehydroascorbic acid, and diketogulonic

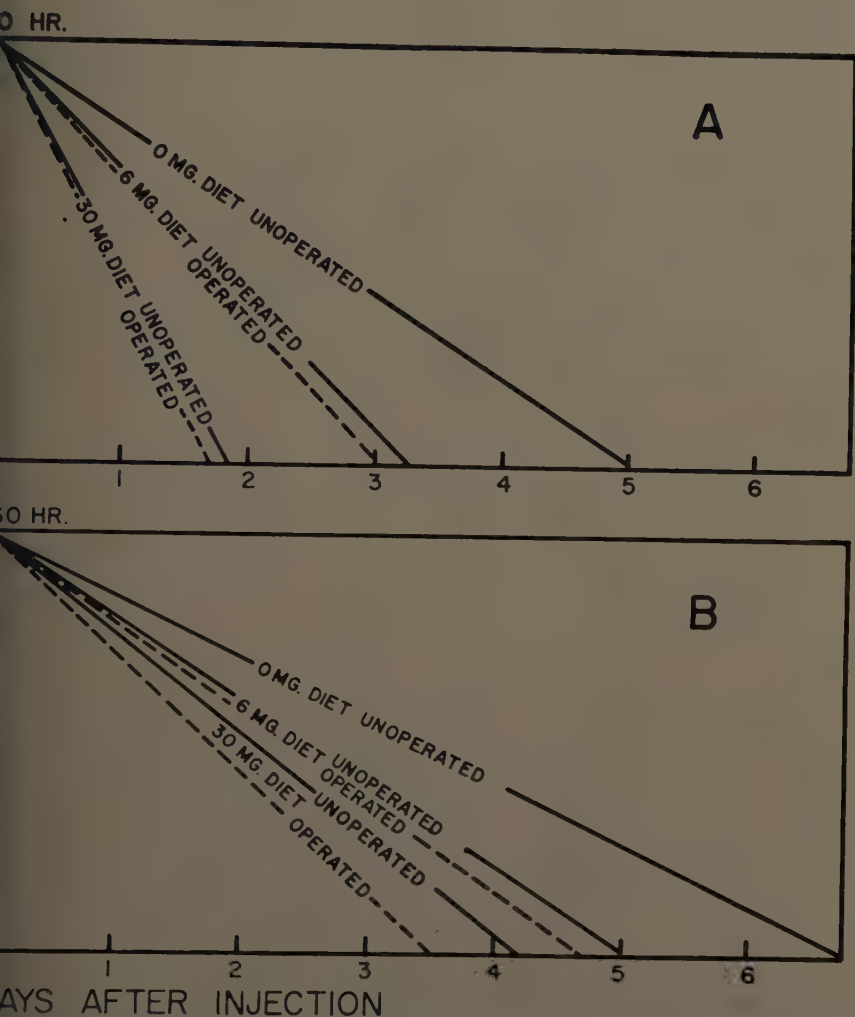


FIGURE 6. Half life in days from 0 and 60 hours before and after operation for 3 groups guinea pigs on 0-mg., 6-mg., and 30-mg. daily ascorbic acid intake.

d in amounts of 70 to 95 per cent 92 hours after injection. Inactive ascorbic d was used as carrier in isotope dilution techniques following the general procedure of Burns *et al.*⁹

From the values of radioactivity remaining in the body we have calculated f lives and shown them to vary according to the previous dietary intake.

The half lives have been calculated both from 0 time and from 60 hours following injection; this latter calculation was used to give ample time for equilibration of the injected radioisotope (FIGURE 6).

We now wish to present results obtained from the same animals following operation. TABLE 3 is a step tabulation of the radioactive carbon dioxide exhaled on all three types of diets following wounding. Data for the group of animals on the 6-mg. and 30-mg. daily ascorbic acid intakes are presented

TABLE 3
CUMULATIVE EXPIRED $C^{14}O_2$ AS PERCENTAGE OF INJECTED ACTIVITY
BEFORE AND AFTER WOUNDING*

Hours	Daily ascorbic acid intake†					
	0 mg.		6 mg.		30 mg.	
	Before operation	After operation	Before operation	After operation	Before operation	After operation
1	0.2	0.1	0.3	0.2	0.6	0.2
2	0.9	0.5	1.3	0.7	3.0	0.8
3	2.0	1.2	2.8	1.5	5.9	1.6
4	2.9	2.1	4.0	2.4	9.2	3.2
5	3.8	3.0	5.5	3.5	11.6	4.8
6	4.5	3.8	6.5	4.5	13.6	6.8
7	5.3	4.7	7.5	5.7	15.7	8.4
8	5.8	5.5	8.4	6.8	17.3	9.9
9	6.4	6.4	9.2	7.9	18.5	11.6
10	6.7	7.0	9.9	9.2	23.1	13.2
12	8.7	9.3	11.8	11.7	24.0	15.3
24	14.0	16.8	16.8	18.5	31.0	27.6
28	15.0	18.0	17.6	19.5	32.0	28.5
32	16.3	19.2	18.7	20.9	33.2	30.6
36	18.4	21.6	20.7	23.0	38.2	34.2
48	22.2	25.0	25.2	27.0	43.3	39.2
60	—	—	28.0	30.0	45.5	42.3
72	—	—	32.7	33.5	48.9	46.7
84	—	—	34.0	35.7	50.5	48.2
96	—	—	36.6	38.0	53.3	50.8

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† Data represent averages from 2 or more guinea pigs.

for 96 hours following injection of the labeled vitamin; for the scorbutic group for 48 hours following injection. After operation and immediately following the injection of ascorbic acid, a peak in specific activity of expired $C^{14}O_2$ also occurred (FIGURE 7). The wounded animal showed a lower $C^{14}O_2$ excretion in the early postoperative than in the corresponding preoperative excretion period. This diminution in $C^{14}O_2$ during the early excretory period in the operated animal coincided with, and we believe accounted for, the increased deposition of ascorbic acid in the scar, noted by chemical and radioactivity determinations. We had previously noted the slight fall in blood level of ascorbic acid following operation. This corresponded with the higher postoperative values of ascorbic acid in the wound tissue (FIGURE 8). Data from FIGURES 7 and 8 further dem

strate that the stress of operation did not accelerate the total metabolism of ascorbic acid.

The total C¹⁴O₂ excretions were approximately equal when measured at 96 hours for the preoperative and postoperative groups. When the total urinary excretion was calculated separately we noted an increased excretion following operation (TABLE 2).

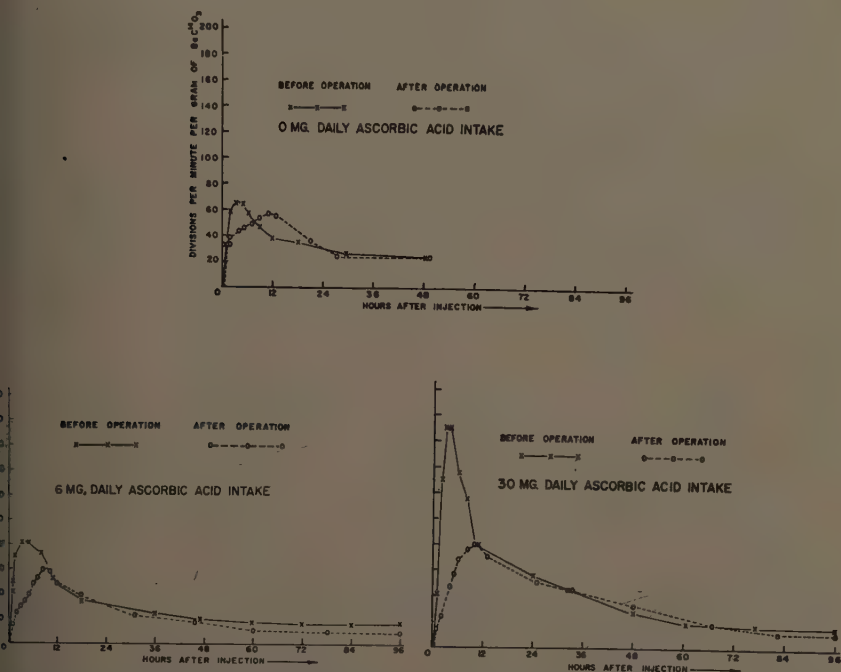


FIGURE 7. Hourly specific activity of expired C¹⁴O₂ before and after operation. Curves for animals on daily ascorbic acid intakes of 0 mg., 6 mg., and 30 mg. Reproduced by permission of the *American Journal of Physiology*.⁵

Rate constants were calculated as

$$\frac{\text{change in amount excreted}}{\text{amount (average) remaining} \times \text{time interval}}$$

us at different time intervals, the rate constants described the slope of a tion of the disappearance curve and were calculated (TABLE 4) following ection of L-ascorbic-1-C¹⁴ acid at 15, 40, and 78 hours from data presented ABLES 2 and 3. It will be noted that for the same guinea pigs the rate onts after operation are slightly below those before operation at 15 hours, al at 40 hours, and slightly higher at 78 hours. The possibility therefore ains that in spite of the fact that there was no systemic increase in metab- m, the tissue comprising the wound may have assumed an independent abolic rate different from that of the animal.

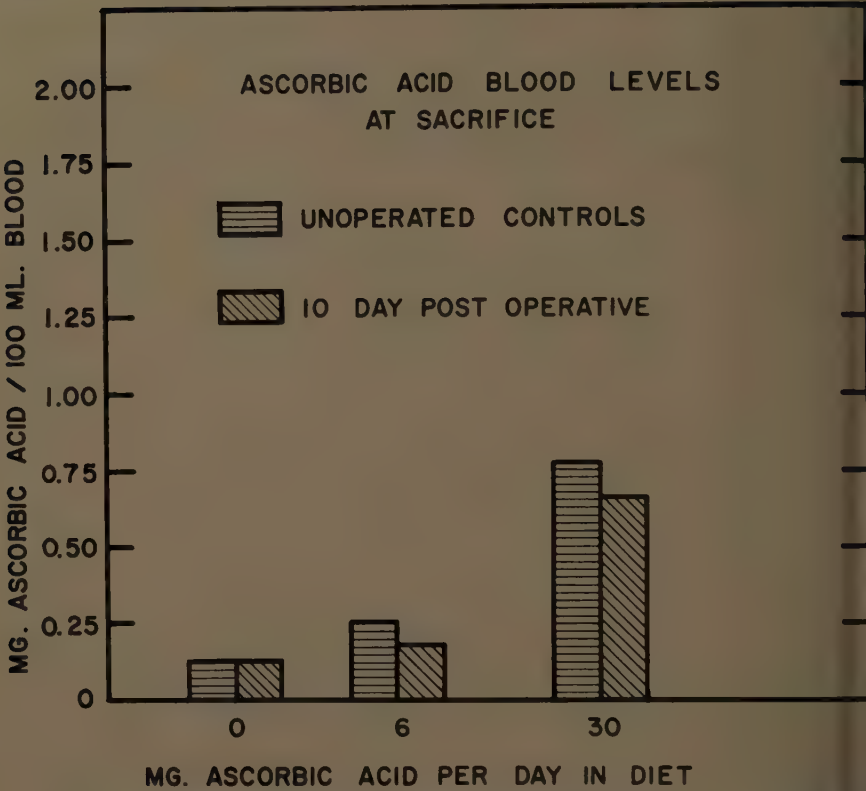


FIGURE 8. Ascorbic acid blood levels of unoperated and operated guinea pigs on different vitamin C intakes. Data taken from groups of 5 animals.

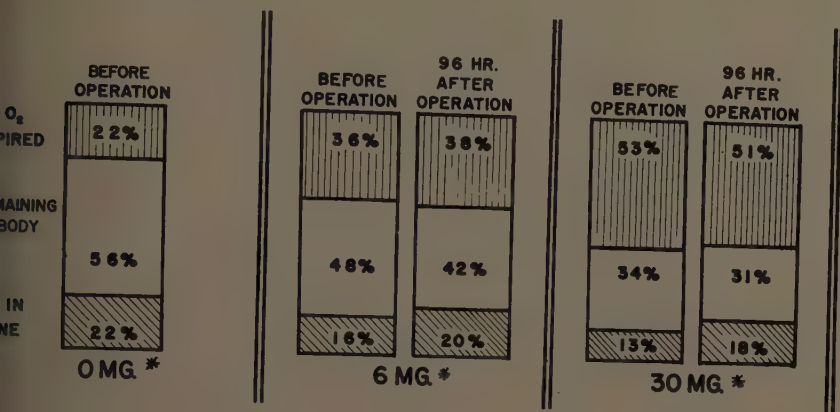
TABLE 4
RATE CONSTANTS FOLLOWING INJECTION OF L-ASCORBIC-1-C¹⁴ ACID*

Hours	Daily ascorbic acid intake†					
	0 mg.		6 mg.		30 mg.	
	Before operation	After operation	Before operation	After operation	Before operation	After operation
	Rate constants					
15	0.254	0.230	0.359	0.355	0.503	0.482
40	0.141	—	0.180	0.177	0.256	0.255
78	0.105	—	0.138	0.158	0.163	0.206

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† Determinations made before and after operation on 3 groups of guinea pigs on 0, 6, and 30 mg. daily ascorbic acid intake.

A graphic representation summarizing the cumulative distribution of ascorbic acid in the guinea pig before and after wounding up to 96 hours following incision is presented in FIGURE 9.



* DAILY ASCORBIC ACID INTAKE

FIGURE 9. Distribution of C¹⁴ activity in expired air, tissue, and urine of guinea pigs for 6-hour period before and after operation. Data are taken from 2 or more guinea pigs.

Conclusion

From the data presented in this paper it may be concluded that tissues depleted of vitamin C need to accumulate and store ascorbic acid in order to perform normal function. It has also been demonstrated that the depleted animal does not systemically metabolize ascorbic acid more rapidly than the animal on adequate vitamin C intake. These statements apply to the intact animal.

Since we have previously shown that ascorbic acid is necessary for both formation and maintenance of the connective tissue comprising the surgical wound, we may further conclude from the data here presented that ascorbic acid accumulated at the wound site in a period shortly following incision when fibrin and blood cells had accumulated before connective tissue could be formed. Furthermore, the systemic metabolism of the wounded animal was quantitatively similar but showed qualitative differences from the intact animal. The diminution in catabolism during the early excretory period in the operated animal accounted for the increased deposition of ascorbic acid in the scar. An independent metabolic rate for the wound tissue per se has never been investigated and could possibly differ from the systemic metabolism of the entire animal.

From our studies on the catabolism of injected radioactive ascorbic acid and derived values for the radioactivity remaining in the body, we have calculated half lives and have demonstrated their variation with the previous dietary intake. The saturated animal showed the shortest half life, the depleted animal the longest.

If the differences in retention in the guinea pig here presented may be applied to the human metabolism of ascorbic acid, the reason for the controversy concerning the daily requirements of vitamin C can be explained. The higher the previous dietary consumption of vitamin C, the lower the retention of additional intake of ascorbic acid. Thus the daily requirement calculated from the excretion of an individual on a saturated diet would be less than the requirement calculated for an individual on a low dietary intake of vitamin C.

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THE BIOCHEMICAL ROLE OF ASCORBIC ACID IN CONNECTIVE TISSUE*

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Scurvy as described in the 17th and 18th centuries was certainly not due simply to acute vitamin C deficiency but to a multifaceted chronic nutritional deficiency.¹ Nevertheless, even then, abnormalities of the connective tissues were prominent among the lesions described. These included nonhealing ulcers and wounds, falling teeth, bone fractures that did not heal, and fractures and wounds that, years after healing, broke open anew.

During the first half of this century, macro- and micromorphologic studies of scurvy and experimental vitamin C deficiency reinforced the view that the primary defect of scurvy resided in the connective tissues. Aschoff, Holst, Röhmlich, Höjer, and Wolbach are among the names associated with these studies.^{2,3} The following conclusions derived from these observations formed the basis for the design of biochemical studies:

- (1) The formation and maintenance of normal collagen require ascorbic acid.
- (2) A nonfibrous, collagen precursor is formed instead of fibrous collagen during an ascorbic acid deficiency.
- (3) Abnormalities of the mucopolysaccharides of ground substance accompany an ascorbic acid deficiency.
- (4) The connective tissue lesions of ascorbic acid deficiency are found preeminently in tissues subjected to physical stress.

Maintenance of Collagen

Initial biochemical studies explored the suggestion that ascorbic acid is necessary for the maintenance of established collagen. Contrary to expectation, I found that the concentration of collagen determined chemically in a variety of organs or tissues, including repair tissue, did not decrease in either acute or chronic scurvy.^{4,5} Similar data were obtained by other investigators.^{6,7} Studies of collagen turnover using isotopes appeared in 1953 also⁸ and led to the conclusion that collagen once laid down is metabolically inert. The biochemical evidence appeared to warrant the conclusion that during scurvy collagen was at least as stable as other proteins and that ascorbic acid is not specifically involved in its maintenance. However, it is now clear that although the great bulk of collagen in most tissues is metabolically inert, certain fractions are highly active and that, in some tissues, a large fraction of collagen is liable to rapid catabolism.^{9,10} The disappearance of as much as 50 per cent of newly laid-down collagen from polyvinyl sponge granulomas, when animals were made scorbutic, has recently been reported by Gould.¹¹ Collagen degradation consequent to ascorbic acid deprivation apparently may occur in collagenous tissues containing an appreciable number of fibroblasts. This will be discussed later, excessive mucopolysaccharides accumulate in these

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cellular tissues during scurvy.¹² The hydrophilic mucopolysaccharides and water inspissate collagen fibers, disaggregating them, thus decreasing the tensile strength of the tissues and probably rendering the collagen more liable to proteolysis.

Two years ago Williams¹³ found that carrageenan, a seaweed polysaccharide, when injected intradermally into a normal guinea pig caused disintegration and disappearance of skin collagen, but that collagen was not destroyed to the same extent if the animals were scorbutic. This case of an ascorbic acid requirement for collagen disappearance, inexplicable as it is interesting, confuses the above reasonably satisfactory picture of the relation of ascorbic acid to collagen maintenance.

Synthesis of Collagen

The biochemical demonstration of an ascorbic acid requirement for collagen synthesis, in contrast to maintenance, was readily demonstrable. Any situation such as wounding, granuloma formation, or tumor growth that leads to a rapid and massive formation of new collagen in adequately fed animals provoked the formation of a collagen-poor tissue in guinea pigs deprived of ascorbic acid. I have found the granuloma induced by subcutaneous injection of carrageenan especially satisfactory for these studies since it affords a large amount of tissue within which collagen formation is very sensitive to the availability of ascorbic acid.¹⁴ Although the total mass and protein content of the granuloma that develops in the guinea pig deprived of ascorbic acid is about the same as in the granuloma from the adequately fed animal, the collagen concentration is very much less. Since the "scorbutic" granuloma develops during only a fortnight of vitamin deprivation, it may be obtained in an essentially normal host that is still gaining weight. The hazards of inanition that plague the interpretation of many experiments involving vitamin C deficiency are eliminated. Essentially similar results demonstrating the need for ascorbic acid in collagen synthesis have been obtained by several investigators.^{15,16} However, even the universality of this conclusion must be questioned. Gould presents evidence elsewhere in this monograph that some collagen, which he refers to as "growth" collagen, may be synthesized even in the absence of demonstrable ascorbic acid. This finding and the implication of earlier histologic studies that only collagen formation in repair tissue required ascorbic acid prompted a study of the question of whether the need for ascorbic acid is determined by the type of collagen synthesis (repair or nonrepair) or whether the potential rate of collagen synthesis is determining.

The choice could not be ascertained by simply determining the effect of ascorbic acid deficiency on the collagen concentrations of normally growing organs. These concentrations do not change during ascorbic acid deprivation because before the vitamin stores are depleted inanition supervenes and growth ceases.⁴

One approach used to study the problem was to measure collagen synthesis in the estrogen-stimulated, involuted uteri of ovariectomized guinea pigs. The average amount of new collagen synthesized in the animals receiving ascorbic acid was 70 mg.; in comparable animals deprived of ascorbic acid it was 9 mg. (unpublished data).

Another study made use of proline- C^{14} . Mammalian collagen contains about 4 per cent hydroxyproline, an amino acid that, while not peculiar to collagen and its derivatives, is seldom found in other proteins.¹⁷ It is commonly used for the identification and quantification of collagen in mammalian tissues. Essentially none of the hydroxyproline in collagen can be derived from free hydroxyproline but must arise from hydroxylation of proline.^{18,19} Determination of the specific activity of hydroxyproline, isolated from the crude collagen fraction of a tissue after administration of proline- C^{14} , may be used as a measure of collagen synthesis. This technique was applied to skin, bones, and liver from guinea pigs deprived of ascorbic acid for only 11 days (unpublished data). These animals did not show the morphologic defects of scurvy at this time, yet it is clear that the synthesis of skin collagen and bone collagen was impaired in the ascorbic acid-deficient animals and was restored by administration of ascorbic acid (TABLE 1). A similar effect on collagen synthesis in liver could

TABLE 1
ASCORBIC ACID AND NONREPARATIVE COLLAGEN SYNTHESIS

Guinea pigs	Specific activity of hydroxyproline*		
	Bone	Skin	Liver
Normal (adequate diet for 14 days)	140	86	450
Ascorbutic (deficient diet for 14 days)	28	4	560
Recovery (deficient diet for 11 days; then 100 mg. ascorbic acid per day)	150	33	560

* Counts per minute per micromole.

Eleven days after beginning the experiment the guinea pigs were injected I.P. with 8 μ c. proline- C^{14} . This was repeated at 12-hour intervals until 48 μ c. had been injected. Twelve hours after the last dose, the animals were killed and hydroxyproline was isolated from the collagen fraction of each tissue.²⁷ The recovery guinea pigs received ascorbic acid only during the period of isotope administration.

was demonstrated only in severe vitamin deficiency. It would appear that ascorbic acid is required for maximal collagen synthesis in a variety of tissues and not merely in repair tissue.

This relation of ascorbic acid to maximal collagen synthesis is clearly seen in the carrageenan granuloma, where the potential rate of synthesis is high (FIGURE 1). Although some collagen has been synthesized even in those granulomas with very low ascorbic acid concentrations, the maximum synthesis was not reached until the tissue contained 40 to 50 μ g. of ascorbic acid per gram (unpublished data).

Action of Ascorbic Acid in Collagen Synthesis

The elucidation of the biochemical roles of ascorbic acid in collagen synthesis requires a knowledge of the site of action of the vitamin. Does it affect synthesis in the connective tissue only indirectly, by influencing the synthesis or release of a hormone, or by maintaining intact the capillary blood supply; or does ascorbic acid act directly in the collagen-synthesizing tissue?

Gould²⁰ adduced evidence for a local action of ascorbic acid when he found

that following an injection of very small amounts of ascorbic acid into one of a pair of sponge granulomas in scorbutic guinea pigs, the hydroxyproline content of the injected sponge was several-fold higher than that of the contralateral sponge.

I have been able to show that *in vitro* addition of ascorbic acid to suspensions of scorbutic carrageenan granuloma incubated with proline- C^{14} increased the specific activity of the isolated collagen hydroxyproline, whereas this was not the case in granulomas from animals that had received ascorbic acid (unpublished data).

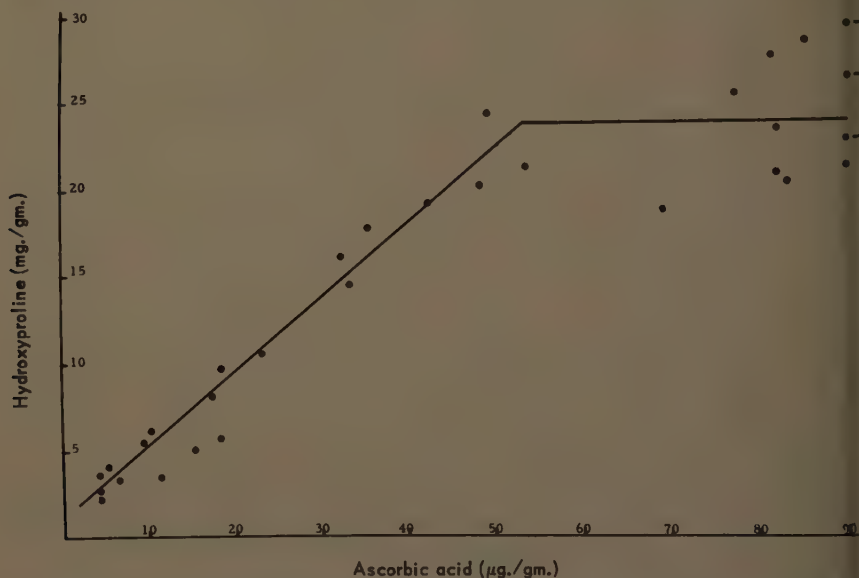


FIGURE 1. The relation of collagen hydroxyproline to ascorbic acid concentration in carrageenan granulomas. Ascorbic acid and collagen hydroxyproline were determined on aliquots of 14-day carrageenan granulomas from guinea pigs that had been given different (0 to 25 mg. per day) but constant ascorbic acid intakes for at least 7 days.

A local action of ascorbic acid on the collagen-forming tissue having been demonstrated, the series of reactions involved in collagen synthesis must be considered in order to specify more explicitly the action of the vitamin. Present evidence indicates that the basic collagen protein, tropocollagen, an asymmetric molecule with a molecular weight of 320,000, is produced intracellularly but aggregates extracellularly, first to fibrils and then to fibers.²¹ Tropocollagen is metabolically very active and is soluble in cold neutral salt solutions but as fibers are formed it becomes metabolically less active and less soluble. All mammalian collagens contain the amino acids hydroxyproline and hydroxylysine. Collagen synthesis requires two reactions in addition to those common to protein synthesis, such as amino acid activation and peptide bond formation. These are hydroxyproline and hydroxylysine synthesis and fiber formation (FIGURE 2).

Fiber formation from the basic collagen molecule takes place *in vitro* nor

zymatically at body temperature. While many compounds can effect the rate of this reaction, ascorbic acid seems to have no peculiar or profound effect.²² Jackson and Bentley (personal communication) have studied this conversion in animals with isotopes and have concluded that the rate of conversion of soluble collagen to fibers is unaffected by an ascorbic acid deficiency. The decreased concentration of soluble collagen in the skin of scorbutic guinea pigs²³ so suggests that the defect of scurvy precedes fiber formation.

Ascorbic acid deficiency, if divorced from the effects of inanition, does not appear to affect activation of amino acids or synthesis of proteins other than collagen. Thus the specific activity of proline in noncollagen proteins after administration of proline-C¹⁴ is not decreased by vitamin C deprivation (TABLE 1). Similar results have been obtained²⁴ with glycine-N¹⁵. Mitoma²⁵ has also shown that the synthesis of an adaptive enzyme is unimpaired in the scorbutic guinea pig. These results, of course, should not be construed to preclude the

TABLE 2
ASCORBIC ACID AND THE SYNTHESIS OF NONCOLLAGEN PROTEINS

Guinea pigs	Specific activity of proline*			
	Serum	Bone	Skin	Liver
Normal	2500	1600	1500	2000
Scorbutic	3700	1700	1500	2500
Recovery	3400	1700	1500	3000

*Counts per minute per micromole.

The same guinea pigs were used as in the experiments reported in TABLE 1. After extraction of the collagen with hot 5.5 per cent trichloroacetic acid, proline was isolated from the hydrolyzates of the residual protein.

possibility that ascorbic acid may affect only synthesis of peptide chains in those cells responsible for collagen synthesis.

The *in vivo* conversion of proline to hydroxyproline is intimately connected with collagen synthesis and since proline, but not free hydroxyproline, is incorporated into the collagen molecule, Stetten¹⁸ suggested that proline was hydroxylated to hydroxyproline only after being built into a peptide bond. Some of our results,¹⁴ as well as those of Gould and Woessner,¹⁵ were in apparent agreement with this hypothesis. It was suggested that during an ascorbic acid deficiency the hydroxylation of proline was blocked and a protein accumulated that in some ways resembled collagen but contained no hydroxyproline. Such a protein might correspond to the nonfibrous collagen precursor described by Wolbach and Howe.²⁶ To test whether, during a deficiency, a protein accumulated that was changed to collagen by administration of ascorbic acid, administered proline-C¹⁴ to guinea pigs bearing scorbutic carrageenan granulomas.²⁷ Large doses of ascorbic acid were given concurrently with the labeled proline to some of the guinea pigs. If there were an accumulation of a proline-rich, hydroxyproline-poor collagen precursor in scorbutic granuloma, the proline would have been built into the precursor before labeled proline was given. One should then have expected the specific activity of the collagen hydroxy-

proline to be low in the scorbutic animals receiving ascorbic acid and proline- C^{14} ; however, the specific activity of the hydroxyproline in these recovery animals was even higher than in normals. Specific activity and collagen synthesis were lowest in unsupplemented scorbutic animals. Last year Gould²⁸ discussed in detail the precursor problem and concluded that although the stage is set for collagen synthesis in scorbutic tissue and collagen rapidly replaces other proteins once ascorbic acid is added, the data indicate that this process does not involve the conversion of an accumulated collagen precursor to collagen.

Not only is there no evidence for the build-up of a collagen precursor in scurvy, but studies of normal collagen synthesis in tissue culture²⁹ and in granuloma slices¹⁹ have failed to yield evidence for even the transient existence of a protein precursor. Instead it appears that hydroxyproline is derived from proline before the latter is built into a protein molecule.

In order to see whether ascorbic acid was specifically involved in the hydroxylation of proline, we examined the specific activity of nonprotein hydroxyproline,²⁷ (that is, hydroxyproline soluble in cold trichloroacetic acid) from granulomas of normal and scorbutic guinea pigs after administration of proline- C^{14} . The lower total activity and the specific activity of hydroxyproline from scorbutic granulomas suggested that a primary defect of hydroxylation exists in scorbutic granulomas. However, in so far as some collagen might have been dissolved by the trichloroacetic acid, these results are inconclusive. Mitoma²⁸ carried out similar experiments using the specific activity of urinary hydroxyproline as a measure of hydroxylation of proline. He obtained no evidence for a specific impairment of hydroxylation of proline during scurvy. More data is needed to settle this important point. The only other evidence bearing on this hydroxylation is in a purely chemical system. Chvapil and Hurych³⁰ reported that ethylenediaminetetraacetic acid (EDTA), ferrous sulfate, hydrogen peroxide, and ascorbic acid hydroxylated proline to hydroxyproline. We confirmed this by using proline- C^{14} . Allen Price, working in our laboratory, found that lysine was hydroxylated to hydroxylysine by a similar system (unpublished data).

In the scheme of collagen synthesis (FIGURE 2) I have indicated the proline that is hydroxylated and incorporated into the collagen molecule by the non-committal designation "active." Possible loci of ascorbic acid activity are indicated; all have been discussed except the transfer of tropocollagen out of the cell, for which no data are available.

Ascorbic Acid and Mucopolysaccharides

In addition to affecting collagen metabolism, ascorbic acid deficiency results in changes of the mucopolysaccharides of the ground substance of connective tissue. Histologic descriptions of these changes are conflicting, having been described as a decrease in mucopolysaccharides,³¹ an increase in mucopolysaccharides,^{32,33} or as a depolymerization of mucopolysaccharides.³⁴ Two biochemical changes have been reported: one, an accumulation of hyaluronic acid in scorbutic repair tissue, and the other, a decreased incorporation of sulfate into mucopolysaccharides.

A comparison of the normal and scorbutic carrageenan granuloma showed

that there is about five times as much hexosamine-and-uronic-acid-containing mucopolysaccharide in the scorbutic granuloma as in the normal. Isolation of this material from scorbutic granulomas indicated that it is essentially all hyaluronic acid.¹² Similar results have been reported in healing wounds.¹⁶

A decreased incorporation of sulfate into the mucopolysaccharides of healing tendons in scorbutic guinea pigs was reported by Kodicek and Loewi,³⁵ while a decreased incorporation of sulfate into the chondroitin sulfate of scorbutic costal cartilage was reported by Reddi and Norström.³⁶ Friberg³⁷ reinvestigated this

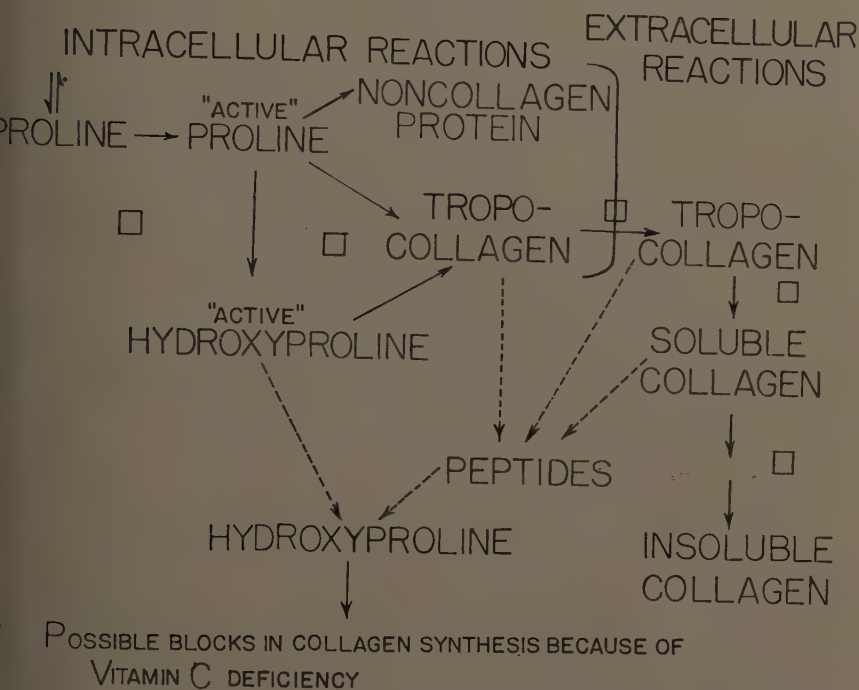


FIGURE 2. Abbreviated scheme relating proline and hydroxyproline to collagen synthesis. Possible sites of ascorbic acid activity are discussed in the text.

problem using paired feeding techniques and showed that ascorbic acid deficiency had no effect on sulfate incorporation into costal cartilage beyond that of inanition. Peyser (personal communication) has studied the effects of acute and chronic scurvy on sulfate in skin. He found no differences in content, uptake, or removal that could be ascribed to ascorbic acid. Hughes and Kodicek³⁸ found the concentration of galactosamine-containing polysaccharides was much lower in scorbutic than in normal granulomas from pair-fed animals. The significance of this finding is that the galactosamine-containing polysaccharides also contain sulfate and that the study was of a rapidly growing tissue. The possible effect of ascorbic acid deficiency on sulfate metabolism in connective tissue must, I think, still be considered an open question.

Nature of Ascorbic Acid Activity

It is clear from the above that although the biochemical changes brought about by an ascorbic acid deficiency have been characterized to some extent, we are still not able to define how ascorbic acid is involved in chemical reactions in the connective cells. In a purely speculative vein I suggest that the activity of ascorbic acid in connective tissue depends on the formation of monodehydroascorbyl and hydroxyl radicals. The existence of free radicals during either the autoxidation or the enzymatic oxidation of ascorbic acid has been postulated several times, with the most conclusive evidence being based on electron paramagnetic resonance studies.³⁹ The hydroxylation of proline to hydroxyproline by a system containing ascorbic acid and hydrogen peroxide and presumably involving a free radical mechanism⁴⁰ has been referred to earlier. If this model hydroxylation had its counterpart in cells and was a rate-limiting reaction, we could expect collagen synthesis to be decreased during an ascorbic acid deficiency. About 20 years ago we showed that ascorbic acid and hydrogen peroxide depolymerized hyaluronic acid,⁴¹ making it more diffusible. A lack of ascorbic acid in tissues might then be expected to permit accumulation of hyaluronic acid by keeping it polymerized and nondiffusible. Since free hydroxyl radical formation *in vivo* would not necessarily involve ascorbic acid, some collagen synthesis might be expected to take place in the absence of ascorbic acid. However, massive or rapid collagen synthesis would require increasing amounts of ascorbic acid. This describes the observed relation of ascorbic acid to collagen synthesis. If the free radical-forming enediol moiety of ascorbic acid determined activity, and if the rest of the molecule determined tissue affinity, vitamin C activity should not require absolute structural specificity. Analogues of L-ascorbic acid, as has been shown, are able to replace the vitamin in preventing scurvy if their concentration in tissues is maintained.⁴² Free radicals are notoriously nonspecific in their attack. The ascorbic acid literature and the papers presented in this monograph attest to the variety of processes in which ascorbic acid may play a permissive if not essential or specific role. Although there is no direct evidence for the free radical behavior of ascorbic acid in connective tissue, the hypothesis pulls together several observations and suggests further experiments, and is eventually susceptible to test.

Summary

The present status of biochemical investigations of the functions of ascorbic acid in connective tissue has been discussed. Maintenance of preformed collagen does not generally require ascorbic acid, but a deficiency of the vitamin may result in loss of collagen from tissues that still contain a large number of fibrocytes.

Small amounts of collagen may apparently be synthesized in the absence of demonstrable ascorbic acid, but the rapid synthesis of large amounts of collagen requires ascorbic acid. Ascorbic acid acts at the cellular level to increase collagen synthesis.

There is no evidence for the accumulation of a nonfibrous, hydroxyproline-poor, protein precursor of collagen when ascorbic acid is lacking. Present evidence, although far from conclusive, suggests that ascorbic acid may be

concerned with the conversion of proline to hydroxyproline before synthesis of the peptide chain.

Lack of ascorbic acid leads to accumulation of hyaluronic acid in repair tissue; the evidence for an effect of ascorbic acid on the metabolism of sulfated mucopolysaccharides is equivocal.

It is suggested that the function of ascorbic acid in connective tissue involves free radical activity.

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ASCORBIC ACID-INDEPENDENT AND ASCORBIC ACID-DEPENDENT COLLAGEN-FORMING MECHANISMS*

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That ascorbic acid plays a definite role in collagen biosynthesis has been amply demonstrated by the fact that after relatively short withdrawal of ascorbic acid from the diets of ascorbic acid-dependent animals, collagen formation in healing wounds and in model systems, as in implanted polyvinyl sponges and in carrageenan granulomas, is markedly impaired. It has also been demonstrated^{1,2} that ascorbic acid is essential to prevent the almost complete resorption of newly formed collagen as studied by the polyvinyl-sponge technique. What, among other things, is not as clear is whether all collagen biosynthesis and maintenance is ascorbic acid-dependent.

In a previous investigation, Woessner and Gould³ found, in a tissue culture study of collagen formation by chick embryo fibroblasts, that collagen was synthesized even in the absence of ascorbic acid. This appeared to be strikingly opposed to the generally accepted concepts and to the subsequent findings by Gould⁴ that indicated a local and direct action for ascorbic acid in collagen biosynthesis. This led to the suggestion that more than one mechanism for collagen synthesis may exist: one such as that involved in normal body collagen formation and another that predominates in tissue repair. The former could be visualized as a slow and stable mechanism relatively independent of ascorbic acid, while the latter would be rapid, perhaps less stable, and ascorbic acid-dependent.

Several other observations point to such a possibility. In earlier work on collagen formation in regenerating skin⁵ and in sponge implants,⁴ it was always found that in the absence of ascorbic acid, collagen formation was markedly impaired but never completely inhibited. It usually was about 15 to 20 per cent of normal. Histological examination always showed a fine collagenous capsule around sponge implants in deficient animals and a dense capsule around sponges in normal animals. Furthermore, while collagen formation in wounds and sponges essentially ceases after six to seven days' withdrawal of ascorbic acid from the diet, young guinea pigs continue to grow at what appears to be a normal rate for a considerable time beyond this depletion period.

It was this last observation that suggested a possible means to test the hypothesis that more than one mechanism for collagen biosynthesis exists and to determine whether there might be both ascorbic acid-independent and dependent mechanisms.

The experiments involved, first, a study of total collagen synthesis in young guinea pigs placed on the scorbutigenic diet from birth compared with collagen synthesis in litter-mates that were given supplements of ascorbic acid. Then after depletion periods, skin wounds were made or polyvinyl sponges implanted

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and collagen formation in these systems could be compared in animals on deficient and supplemented diets to determine whether similar or different mechanisms from those operating in growth were involved. The data to be presented point quite definitely to the possibility of more than a single mechanism and suggest that both ascorbic acid-independent as well as ascorbic acid-dependent mechanisms exist.

Methods

Animals. Newborn animals, weighing from about 60 to 80 gm. were used in most cases. The young animals were placed on a scorbutigenic diet immediately. Litters were divided so that experimental animals were compared with litter-mates whenever possible. Animals were fed by hand at the outset, but they soon fed themselves. Animals that refused the diet or gained no weight were discarded. Animals rarely showed any signs of apparent scurvy before the 16th day and rarely died before the 30th day. Normal controls were fed the scorbutigenic diet supplemented daily with 10 mg. of L-ascorbic acid given orally. In most instances animals were maintained on the scorbutigenic diet for 6 days before wounding⁵ or before implanting the polyvinyl sponges.⁴ Wounds were usually made by excising $\frac{3}{4}$ -in. circles of skin bilaterally and, generally, four 10-mg. sponges were implanted, two on each side of the animal.

Collection of tissue. For total collagen formation, the collagen content of the skin and that of the carcass were determined separately. The hair was removed by plucking as completely as possible, and the entire skin was removed, weighed, frozen in dry ice, and ground in an electric meat grinder. The ground skin was then extracted repeatedly in the cold with 0.45 *M* NaCl to remove salt-soluble collagen. The carcass, minus the head, a long bone, and a small piece of costochondral junction needed for histological examination, was weighed, minced, and then blended in cold 0.45 *M* NaCl and repeatedly extracted to remove salt-soluble collagen.

Regenerating tissue of skin wounds was isolated by careful dissection, and sponges were removed by forceps through a small incision, using care not to include preformed collagenous tissue or the capsule that usually surrounds the sponge.

When *total collagen* hydroxyproline was to be determined, the tissue was dried in acetone for 24 hours, minced finely, and re-extracted with acetone for another 24 to 48 hours, then defatted with ethyl ether, and dried at 108° C. *vacuo*.

Soluble collagen. After each 24-hour shaking period in the cold, the salt extract was collected by centrifugation according to the method described by Gross,⁶ and the tissue re-extracted in the same way five to six times. The combined clear extracts were then dialyzed in the cold against large volumes of distilled or tap water to remove salt and low-molecular-weight compounds. The dialyzed material composed of precipitate and suspending fluid was homogenized, and an aliquot was taken for collagen analysis.

Insoluble collagen. The residue after the extraction with 0.45 *M* NaCl was suspended in water and treated as follows:

1. *Isolation of collagen.* The collagen in the acetone-treated sponge or

tissue, in the dialyzed soluble extract, or in the residue containing the insoluble collagen was converted to gelatin by autoclaving twice for 3 hours each time at 25 psi steam pressure. The extracts were combined and dried in a steam bath in a current of air.

2. *Hydrolysis of the gelatin extract.* The dried autoclave-extractable material was hydrolyzed in 6 *N* HCl in a sealed tube by heating at 150° C. for 3 hours. The hydrolyzate was adjusted to neutrality either by adding the theoretical amount of NaOH or by removing the HCl by repeated evaporation.

Hydroxyproline determination. In view of the essentially unique occurrence of hydroxyproline in collagen, it can be used as a measure of the collagen content. The hydroxyproline content multiplied by 7.46 may be used as a measure of the apparent collagen content. The method employed was that of Neuman and Logan⁷ as modified by Martin and Axelrod.⁸

Experimental

Growth studies. Several litters of newborn guinea pigs were divided into groups so that litter-mates could be maintained on both ascorbic acid-supplemented and ascorbic acid-free diets for varying periods of time. Growth was determined by weighing the animals each morning before feeding. Particular attention was paid through the first 14 days that covered the period involved in subsequent wounding and implantation experiments. A certain number of animals, on both the supplemented and scorbutogenic diets, that refused to eat were discarded.

It is obvious from FIGURE 1 that during the first two weeks after birth the growth rate of guinea pigs, on the diet provided, was essentially independent of added ascorbic acid. Beyond 15 or 16 days it was found that the animals on the unsupplemented diet no longer gained weight as did those receiving ascorbic acid, but they continued to appear in good health for as long as 25 days. Some animals on the scorbutogenic diet subsequently lost considerable weight, but they were generally among those that during the earlier period had gained the most.

Total collagen of animals on scorbutogenic and ascorbic acid-supplemented diets. A large number of animals maintained on the scorbutogenic diet and others that received a daily supplement of ascorbic acid for periods of 15 days or more and that had attained weights of 150 gm. or more were analyzed to determine both the total soluble and insoluble collagen content of the animal. A series of newborn animals were analyzed directly after birth to indicate the base level. Another series was analyzed after six days on the scorbutogenic diet on the assumption that a certain amount of synthesis might occur as a result of ascorbic acid carried over from the mother. A six-day period has been found adequate to deplete the tissues enough to prevent collagen synthesis in ascorbic acid-dependent systems.

An examination of TABLE 1 indicates quite clearly that even in the absence of ascorbic acid young guinea pigs during growth were able to synthesize very substantial amounts of collagen. Only a small fraction was formed during the first six days after birth when there may still have been some residual ascorbic acid present. The rate of synthesis during this period appears to be no greater than during the later period. The total collagen synthesis appears to be di-

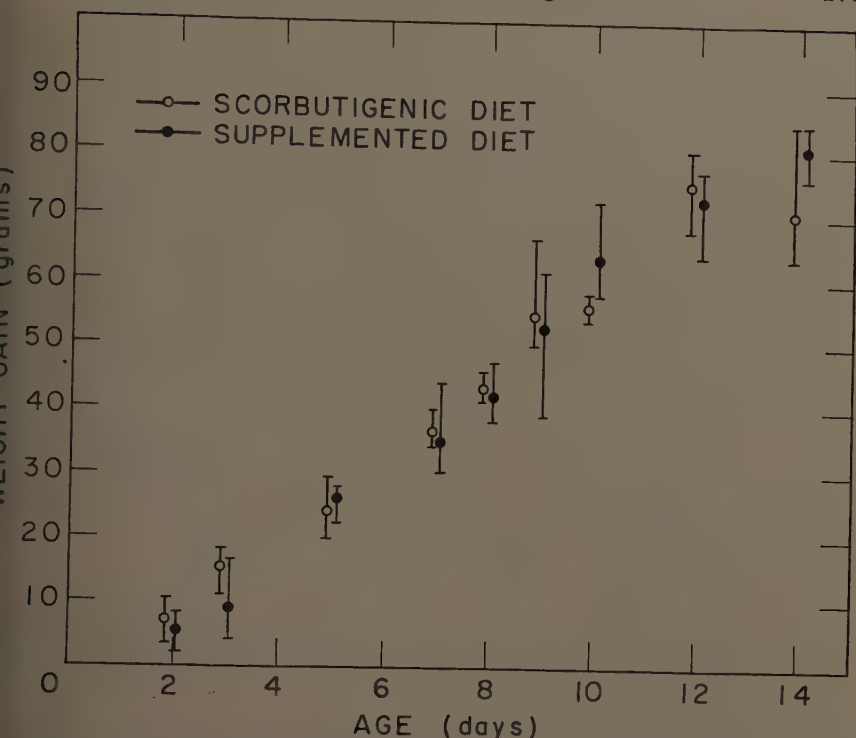


FIGURE 1. Growth of newborn guinea pigs maintained on either a scorbutogenic- or ascorbic acid-supplemented diet. Each point represents the average weight gain of three animals, and the bar represents the range. Each pair of points represents three pairs of litter-mates.

TABLE 1
CHANGES IN TOTAL COLLAGEN CONTENT OF NEWBORN GUINEA PIGS
MAINTAINED ON NORMAL AND SCORBUTIGENIC DIETS

	Skin			Carcass		
	Newborn	Supple- mented	Scorbu- togenic	Newborn	Supple- mented	Scorbu- togenic
total collagen (mg.)	471	1390	928	740	1482	1305
collagen increase/animal (mg.)	—	922	457	—	742	565
collagen increase (mg.)/gm. tissue	—	73.8	83.0	—	10.1	9.7
collagen (mg.)/gm. tissue*	34	52.6	47.6	18.5	13.1	13.3
collagen (mg.)/gm. body weight	6.0	6.4	5.3	9.5	6.8	6.8
soluble collagen (mg.)/gm. insoluble collagen	56.4	159	17.1	17.0	31.8	19.2

Animals were maintained on the scorbutogenic or supplemented diet for 14 to 31 days, all weighed over 150 gm. Average weight of those on scorbutogenic diet was 173 gm. on the supplemented diet 217 gm.

After 6 days on scorbutogenic diet, total collagen of skin and carcass was 517 mg. and 840 mg., respectively.

*Either skin or carcass.

rectly related to the amount of growth. Collagen formation in the carcasses of animals on normal and scorbutogenic diets is strikingly similar, and the formation in the skin is slightly higher in animals on the supplemented diet as compared with animals on the unsupplemented diet.

It is quite apparent that salt-soluble collagen was abundant only in the skin of animals on the supplemented diet. However, even though the animals of the scorbutogenic diet had skins relatively poor in salt-soluble collagen, synthesis occurred quite extensively. Rapid collagen synthesis in the carcass of animals either on ascorbic acid-supplemented or scorbutogenic diets was unaccompanied by the accumulation of high concentrations of salt-soluble collagen. There was a slightly higher concentration in the carcasses of normal animals on the supplemented diet, but it was only about one fifth of the relative amount found in the skin of the same animals.

TABLE 2
COLLAGEN FORMATION IN REGENERATING SKIN WOUNDS IN NEWBORN GUINEA PIGS

Days on scorbutogenic diet		Ascorbic acid* after wounding (days)	No. of animals	Collagen† per cent dry weight
Before wounding	After wounding			
0	5	0	9	11.0 ± 2.7
0	6	0	5	31.7 ± 1.1
6	0	6	5	30.6 ± 2.2
6	6	0	4	5.6 ± 1.4
10	6	0	7	4.5 ± 1.0
10	8	0	4	5.4 ± 0.3
12	6	0	3	6.4 ± 1.1

* Ten mg. per day.

† Calculated from total hydroxyproline $\times 7.46$.

These data make it difficult to interpret the significance of large accumulations of salt-soluble collagen. It does not negate the possibility that salt-soluble collagen is the immediate precursor of the fibrous collagen, but it does suggest that in the skin, during rapid growth under normal conditions, soluble collagen may accumulate more rapidly than it can be fibrillated.

Formation of collagen in skin wounds in growing young guinea pigs maintained on scorbutogenic and ascorbic acid-supplemented diets. A number of groups of newborn animals were wounded by removing circles of skin, each three-fourths inch in diameter. In order to determine whether, as had been reported previously for older animals,⁵ a depletion period was essential before collagen formation was impaired in newborn animals, one group was wounded on the day of birth and continued on the scorbutogenic diet for 5 and 6 days. When collagen synthesis in this group was compared with a control group that had received an ascorbic acid supplement daily (TABLE 2) it was evident that in all probability these newborn carried over sufficient ascorbic acid from the mother to continue synthesis for a short time.

Another group wounded after a depletion period of six days and then maintained on the scorbutogenic diet no longer formed very substantial amounts of

collagen. Other groups wounded after more prolonged preparative periods also formed only a small amount of collagen. It is apparent therefore that even though the animals in these groups could synthesize substantial amounts of "growth" collagen they had been unable to accumulate "repair" collagen effectively except when ascorbic acid was administered.

It is interesting that wounds that could not repair properly still accumulated small but significant amount of collagen. It has been assumed in the past that this is collagen that infiltrates the area from the adjacent tissue. It is possible that this small but significant amount of collagen is the product of an ascorbic acid-independent collagen-synthesizing mechanism. It is also possible that this may serve as a foundation upon which the ascorbic acid-dependent collagen-forming mechanism deposits its rapidly produced collagen. This may explain why collagen formation is so rapid in a wound in a deficient animal after ascorbic acid is administered when compared to the formation

TABLE 3

COLLAGEN FORMATION IN SUBCUTANEOUSLY IMPLANTED POLYVINYL SPONGES
IN NEWBORN GUINEA PIGS

Treatment	Days					
	8	10	11	12	13	14
	Collagen/sponge (μ g.)					
Ascorbic acid-supplemented diet	165	832	938	1125	1130	1365
Scorbutigenic diet	60	173	173	318	338	135

Collagen is based on the hydroxyproline content $\times 7.46$.

Each value is on the average based on 6 animals.

Animals on the supplement received 10 mg. L-ascorbic acid daily.

a normal animal where the rapid repair phase is preceded by an induction period. These possibilities are presently under investigation.

Formation of collagen in polyvinyl sponges implanted in newborn guinea pigs maintained on scorbutigenic and ascorbic acid-supplemented diets. Several series of newborn guinea pigs were placed on the scorbutigenic diet for a period of 10 days, at which time four 10-mg. sponges were implanted subcutaneously in each animal. The animals were divided into two groups so that litter-mates could serve as controls. The animals in one group were maintained on the scorbutigenic diet, and the others received 10 mg. L-ascorbic acid daily. It is evident from TABLE 3 that the animals on the supplemented diet produced an abundance of collagen in the sponges, whereas the animals on the scorbutigenic diet produced considerably less. It is interesting to point out that in the latter some collagen was produced with a tendency for a peak at about 10 days. It is not at all improbable that the small amounts of collagen found when the animals were maintained on the scorbutigenic diet were due to a complex interaction involving both collagen formation and collagen resorption and that in the absence of ascorbic acid resorption kept pace with synthesis.

However, it is clear that the repair mechanism must differ from the mechanism during growth since these animals, during this period of ascorbic acid deprivation, were actively producing growth collagen in the skin and in the carcass.

Summary

The hypothesis that there might be more than one mechanism for collagen biosynthesis, one that is involved in normal body collagen formation and another that predominates in tissue repair, has been tested.

Using newborn guinea pigs it has been demonstrated that body collagen both in the skin and in the carcass, was extensively formed even when the animals were maintained on a scorbutogenic diet, suggesting that such "growth" collagen is essentially ascorbic acid-independent.

On the other hand, collagen formation in skin wounds or in subcutaneously implanted polyvinyl sponges in these same animals was markedly impaired when the animals were deprived of ascorbic acid, suggesting that "repair" collagen is ascorbic acid-dependent.

Even under the most drastic conditions of ascorbic acid deprivation some collagen was formed in wounds and sponges, and it is suggested that both growth and repair collagen are involved in the repair process and that the former may serve as a foundation upon which the rapidly forming repair collagen accumulates.

There remains the possibility that ascorbic acid is involved in the formation of growth collagen but at a level that is extremely small compared with that for repair collagen.

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THE ROLE OF ASCORBIC ACID IN TYROSINE METABOLISM

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It has been known for many years that animals and man deficient in ascorbic acid metabolize tyrosine incompletely when given extra amounts of this amino acid. In fact, the need for vitamin C to maintain normal tyrosine oxidation has been referred to many times as one of the best examples of a requirement for this vitamin in a specific biochemical reaction.

This paper will review briefly the historical development of our knowledge concerning the relationship between ascorbic acid and tyrosine metabolism. Data supporting our present concept of the role of the vitamin in tyrosine metabolism and an interpretation of these results in connection with the general problem of understanding other biochemical and physiological functions of this vitamin will also be given.

The first evidence that ascorbic acid is needed for the complete metabolism of tyrosine was obtained by Sealock and Silberstein in 1939¹ and 1940.² These investigators observed that scorbutic guinea pigs fed L-tyrosine excreted homotartaric acid, *p*-hydroxyphenylpyruvic acid, and *p*-hydroxyphenyllactic acid. However, the excretion of these aromatic acids ceased when L-ascorbic acid was given to these animals. A similar defect in tyrosine metabolism in infants was reported by Levine *et al.* in 1939³ and 1941.^{4,5} Premature infants on a relatively high intake of protein were found to excrete *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid, and full-term infants excreted the aromatic acids when given extra tyrosine or phenylalanine. The defective metabolism in these infants was also corrected by the administration of vitamin C. It is of interest that the defect in scorbutic guinea pigs could be corrected not only by ascorbic acid but, as Woodruff and Darby reported,⁶ by the administration of relatively large amounts of folic acid. Further data concerning the relationship of folic acid and ascorbic acid will be presented in this paper. One of the most important papers concerning the role of ascorbic acid in tyrosine metabolism is that of Painter and Zilva,⁷ who studied the quantitative relationship between the amount of vitamin C required to prevent the excretion of tyrosyl metabolites as the amount of tyrosine fed to guinea pigs was increased. Two findings of particular interest in their paper were: (1) scorbutic guinea pigs on a moderate intake of dietary tyrosine did not excrete tyrosyl metabolites; that is, the defect appeared only upon feeding *extra* tyrosine to the guinea pigs; and (2) when large amounts of tyrosine were fed to scorbutic guinea pigs, the amount of ascorbic acid required to prevent tyrosyluria greatly exceeded the amount of the vitamin necessary to prevent scurvy. Painter and Zilva, with keen insight, questioned whether ascorbic acid was acting as a vitamin in maintaining normal tyrosine oxidation. They concluded,⁷ "The dependence of complete degradation of *l*-tyrosine in high doses on the presence of excess *l*-ascorbic acid in the guinea-pig system does not necessarily indicate a connexion between the normal function of *l*-ascorbic acid and the normal metabolism of aromatic amino-acids."

The study of the relationship between ascorbic acid and tyrosine metabolism *in vitro* proceeded slowly, and most of the work has been done within the last 10 years. Studies in this area had to await the availability of methods making it possible to assay the individual enzymatic steps of tyrosine metabolism. Early attempts to measure tyrosine degradation *in vitro* were discouraging because the amount of tyrosine metabolized was small. Addition of ascorbic acid to these preparations had no effect. In retrospect, it is clear that the main deterrent to progress was the misconception that the initial step in tyrosine catabolism was an oxidative deamination. In 1951⁸⁻¹⁰ several laboratories presented evidence that the initial step was instead, transamination with α -ketoglutarate. Once this fact was realized and liver preparations were supplemented with α -ketoglutarate, progress in studying tyrosine metabolism rapidly followed, and the role of ascorbic acid could be evaluated *in vitro*.

Tyrosine Metabolism in Mammalian Liver

The pathway of tyrosine oxidation, as believed to take place at the present time, is illustrated in FIGURE 1. The first step is a transamination with α -ketoglutarate to yield *p*-hydroxyphenylpyruvic acid. Pyridoxal phosphate is the coenzyme, and neither pyruvate nor oxalacetate can replace α -ketoglutarate.

The next enzyme, *p*-hydroxyphenylpyruvic acid oxidase, is the one with which ascorbic acid participates. The enzyme catalyzes the oxidation of *p*-hydroxyphenylpyruvic acid to homogentisic acid and is present in the liver of all mammalian species thus far examined, and the enzyme has been purified from beef,¹¹ pig,^{11,12} and dog liver.¹³⁻¹⁵ Oxidation of *p*-hydroxyphenylpyruvic acid to homogentisic acid requires 2 atoms of oxygen with the liberation of 1 molecule of carbon dioxide. The reaction is complicated since it involves hydroxylation of the aromatic ring, migration of the side chain, and an oxidative decarboxylation of the pyruvate to an acetate side chain.¹⁶ Recent experiments¹⁷ employing O¹⁸ indicate that at least 1 atom of oxygen taken up is derived from the air. The origin of the second atom of oxygen remains in doubt due to rapid exchange with the solvent.

Until recently it was thought that 2,5-dihydroxyphenylpyruvic acid was an intermediate in this oxidation. Rather conclusive evidence against this assumption is the finding that 2,5-dihydroxyphenylpyruvic acid is not oxidized to homogentisic acid when incubated with human liver homogenates¹⁸ or with purified *p*-hydroxyphenylpyruvic acid oxidase.^{11,13}

p-Hydroxyphenylpyruvic acid oxidase has an optimal pH range between 6.3 and 7.8,¹³ and the optimal conditions for studying its activity manometrically have been determined.^{13,14} The enzyme activity can also be determined spectrophotometrically by observing the rate of disappearance of the enol-borate complex of *p*-hydroxyphenylpyruvic acid.¹⁹ A need for catalase in the manometric assay²⁰ has been attributed to its protective effect on the enzyme.¹¹ However, since small amounts of horse-radish peroxidase can replace the need for catalase, and since the amount of peroxidase employed does not decompose hydrogen peroxide to any significant extent (Zannoni and La Du, unpublished observations), the exact role of catalase in this oxidation is still not clear.

p-Hydroxyphenylpyruvic acid oxidase can be inhibited by low concentrations

f diethyldithiocarbamate¹³ and by sulfhydryl-binding agents such as *p*-chloro-mercuribenzoic acid.¹¹ In view of the known affinity of diethyldithiocarbamate for copper, a search has been made for this metal in the purified enzyme, but there is no evidence that the purified enzyme is a copper-containing protein.^{11,13}

PATHWAY OF TYROSINE METABOLISM IN LIVER

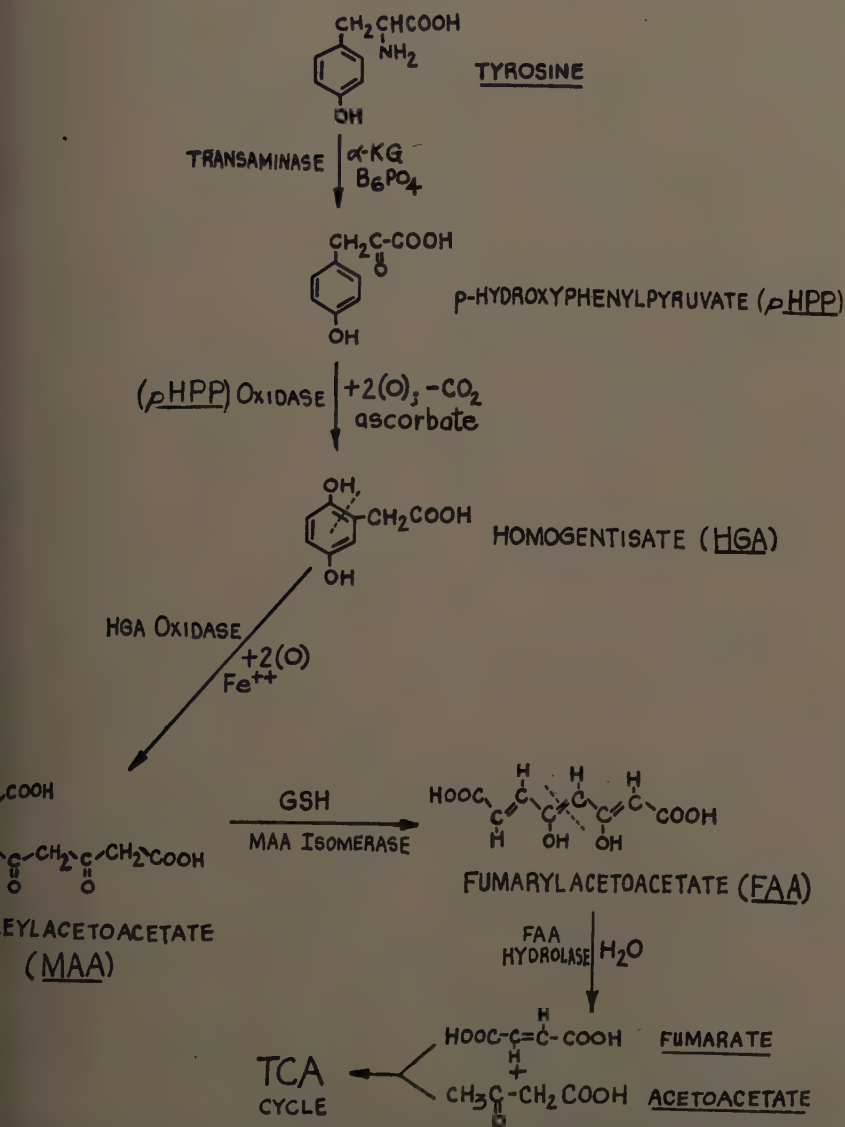


FIGURE 1. Present pathway of tyrosine metabolism in mammalian liver. The following abbreviations are used: B_6PO_4 , pyridoxal phosphate; $\alpha\text{-KG}$, α -ketoglutarate; GSH, glutathione.

p-Hydroxyphenylpyruvic acid oxidase is highly specific for its substrate and does not catalyze the oxidation of other hydroxyphenylpyruvic acids or a number of related compounds.^{11,13} The Michaelis-Menten constant for *p*-hydroxyphenylpyruvic acid with the oxidase has been found to be $4.2 \times 10^{-5} M^{11}$ (or $2.0 \times 10^{-5} M^{21}$). The oxidation has been reported by Hager *et al.*¹¹ to have an unusual temperature coefficient of 6.2, but in our laboratory repeated determinations yield values of approximately 2.0 per 10° temperature rise between 25° and 37° C.

p-Hydroxyphenylpyruvic acid oxidase is inhibited by a number of structurally related analogues of *p*-hydroxyphenylpyruvic acid. Salicylaldehyde and *p*-hydroxybenzaldehyde also inhibit in relatively high concentrations.¹⁵ In general, compounds with a phenyl group and a side chain containing a carbonyl group are inhibitors of *p*-hydroxyphenylpyruvic acid oxidase. It is of interest that 2,5-dihydroxyphenylpyruvic acid has no inhibitory effect at concentrations equal to the substrate. Phenylpyruvic acid is the most potent inhibitor of the compounds tested. Preincubation of *p*-hydroxyphenylpyruvic acid oxidase for 1 min. with $1 \times 10^{-5} M$ phenylpyruvate in the presence of oxygen caused up to 80 per cent inhibition.¹⁵

The observation of Sealock and Silberstein² that scorbutic guinea pigs excreted homogentisic acid as well as *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid suggested that ascorbic acid was involved in the oxidation of homogentisic acid as well as the oxidation of *p*-hydroxyphenylpyruvic acid. However, Suda *et al.*^{22,23} demonstrated that ferrous iron was required for homogentisic acid oxidase activity and that the only effect of ascorbic acid in this step was as a nonspecific reducing agent to maintain iron in the ferrous form.

Effect of Ascorbic Acid on Tyrosine Metabolism in Vitro

An early example of the stimulatory effect of ascorbic acid on tyrosine oxidation *in vitro* is shown in FIGURE 2. Here the oxidation was followed manometrically, using an extract of rat liver acetone powder as the source of enzymes which shows that ascorbic acid does not stimulate the oxidation of L-tyrosine if added alone. However, if ascorbic acid is added with α -ketoglutarate, the latter being necessary to accept the amino group from tyrosine in the transaminase reaction, ascorbic acid then has a definite stimulatory effect.

Another aspect of the stimulatory effect of ascorbic acid is perhaps shown more clearly in FIGURE 3. The effect of the vitamin is not on the initial rate of oxygen uptake but rather to maintain the initial rate of oxidation for a longer time. Without ascorbic acid, the reaction slows down and stops after 5 to 10 min.; with ascorbic acid, it continues at the initial rate until the substrate is completely oxidized to acetoacetic acid.

L-Ascorbic acid is not specifically required to maintain tyrosine oxidation *in vitro*, and the proposal advanced by Sealock and Goodland²⁶ that vitamin C was a specific coenzyme for *p*-hydroxyphenylpyruvic acid oxidase was not supported by further work. The ability of a number of compounds to replace ascorbic acid in this reaction is summarized in TABLE 1. As may be observed many compounds structurally unrelated to ascorbic acid, although possessing

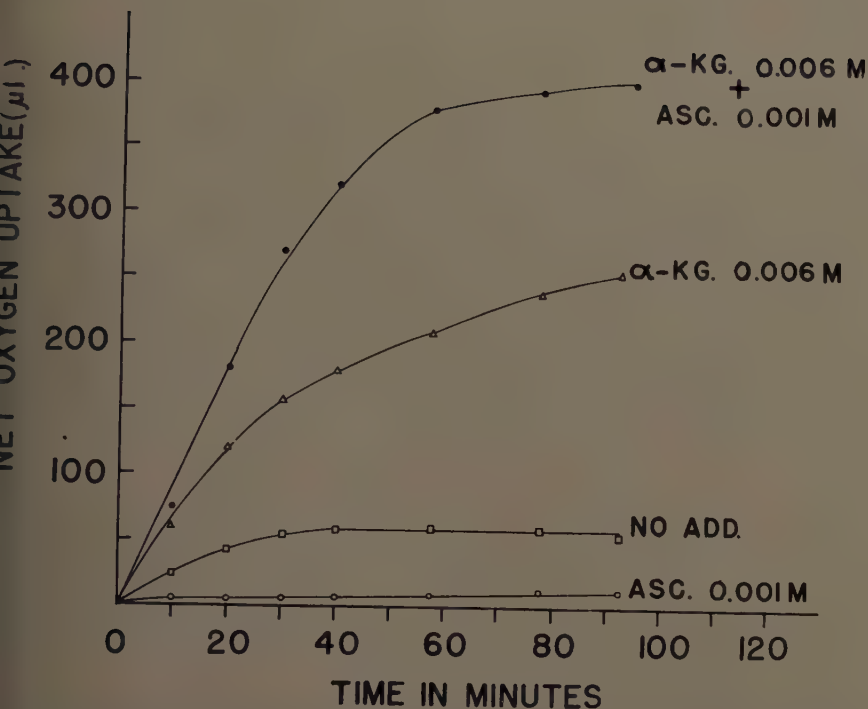


FIGURE 2. The stimulation of L-tyrosine oxidation by ascorbic acid (ASC) in the presence of α -ketoglutarate (α -KG). Flasks contained 10 μ moles of L-tyrosine.²⁴ Reproduced by permission from *Science*.

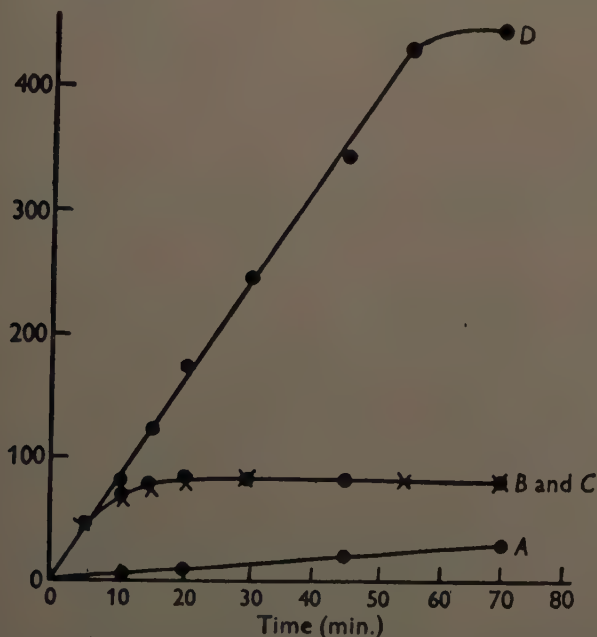


FIGURE 3. The effect of ascorbic acid on the oxidation of L-tyrosine. All flasks contained α -ketoglutarate and the soluble dialyzed enzyme. Curve A, ascorbic acid; Curve B, tyrosine; Curve C, tyrosine plus glutathione; and Curve D, ascorbic acid and tyrosine. Reproduced by permission of Johns Hopkins Press.²⁵

similar redox properties, also are effective in maintaining tyrosine oxidation. One of the most active compounds in this regard is the dye, 2,6-dichlorophenol-indophenol.

How do these compounds maintain tyrosine oxidation? The primary effect of these reducing agents is to prevent an unusual type of inhibition of *p*-hydroxyphenylpyruvic acid oxidase by its substrate, *p*-hydroxyphenylpyruvic acid. This inhibition is illustrated in FIGURE 4. In these experiments, using different amounts of substrate, it can be seen that with 2.5 and 5.0 μ moles of *p*-hydroxyphenylpyruvic acid, there is complete oxidation. However, with larger amounts of substrate, less and less is metabolized, so that with 15 μ moles only about 2.5 μ moles of it are metabolized before oxidation stops. We note

TABLE 1
ACTIVATION OF L-TYROSINE OXIDATION

	Rienits	Painter and Zilva	Knox and Knox	Sealock <i>et al.</i>	La Du and Greenberg
Ascorbic acid	+	+	+	+	+
Isoascorbic acid	—		+	+	+
Glukoascorbic acid	—	+		+	+
Dichlorophenolindophenol					+
Hydroquinone					+
Reductone				+	
Dihydroxymaleic acid			—		—
Glutathione			—	—	—
Cysteine			—		—
H ₂ O ₂			—		
Catechol					—
DOPA					—
2-Methyl ascorbic acid				—	
2,3-Dimethyl ascorbic acid				—	
Pteroylglutamic acid	+		—		

Summary of the types of compounds tested for their ability to replace ascorbic acid in the oxidation of L-tyrosine by cell-free liver preparations. Key: +, effective; —, ineffective; blank, not tested. Reproduced by permission of the Johns Hopkins Press.²⁵

that the *initial rate* of oxidation is about the same at all concentrations of substrate used in this experiment.

What causes the slowing down of the reaction rate when larger amounts of substrate are used? Is it due to an inactivation of the enzyme that accompanies enzymatic activity as seems to be true for catalase,²⁷ tyrosinase,²⁸ and ascorbic acid oxidase?²⁹ (See also C. R. Dawson and K. Tokuyama's paper in this publication.) This possibility has been examined, and it has been shown that catalytic activity alone does not inactivate the enzyme (FIGURE 5). If 15 μ moles of *p*-hydroxyphenylpyruvic acid are added to the enzyme preparation in three 5- μ mole portions, waiting until each portion has been completely oxidized before the next is added, the enzyme is not inhibited and the substrate is completely oxidized. However, if 10 μ moles of substrate are added at one time with the same amount of enzyme as used previously, the enzyme becomes inhibited and further substrate is not oxidized. Thus it is not how much sub-

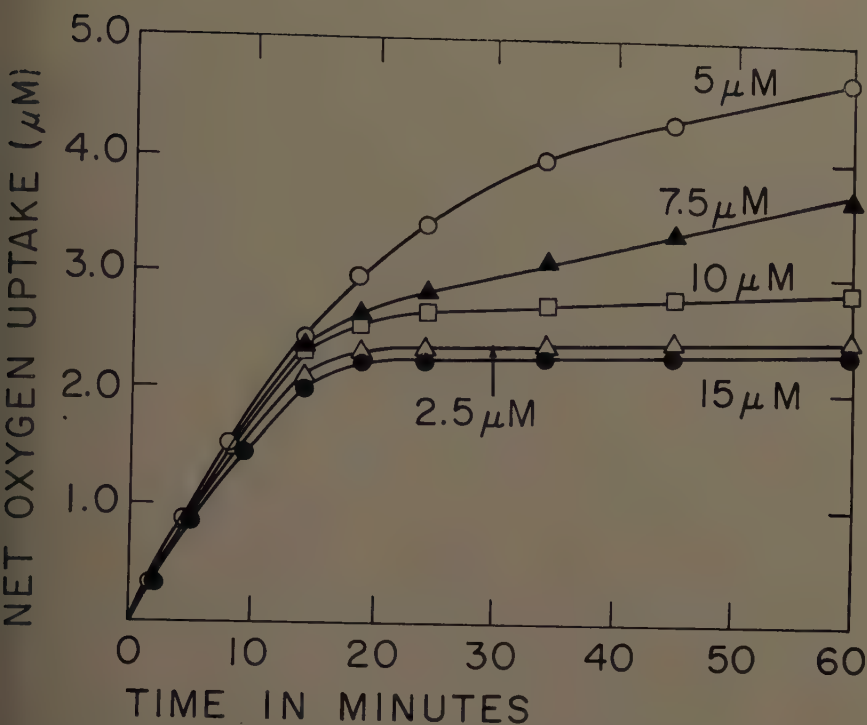


FIGURE 4. Oxidation of various concentrations of *p*-hydroxyphenylpyruvic acid.¹³ Reproduced by permission from *The Journal of Biological Chemistry*.

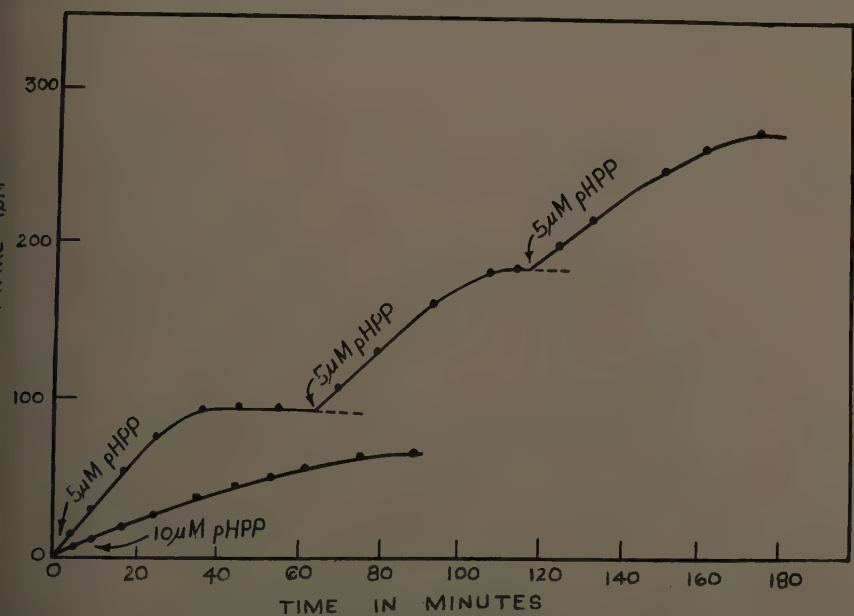


FIGURE 5. Oxidation of *p*-hydroxyphenylpyruvic acid (pHPP) added at various times indicated (\downarrow). See text for details.

strate has been oxidized but rather the high concentration of substrate present that leads to inactivation. Recently evidence has been obtained suggesting that excess substrate leads to inactivation of the enzyme through the formation of small amounts of an inhibitory product that does not form when lower substrate concentrations are used.¹⁵ However, the exact mechanism of enzyme inhibition is still uncertain, and other possibilities must also be considered.

The protective effect of reducing agents such as 2,6-dichlorophenolindophenol in preventing substrate inhibition is shown in FIGURE 6. In this experiment, 20 μ moles of *p*-hydroxyphenylpyruvic acid were incubated in each Warburg vessel with various amounts of reduced 2,6-dichlorophenolindophenol.

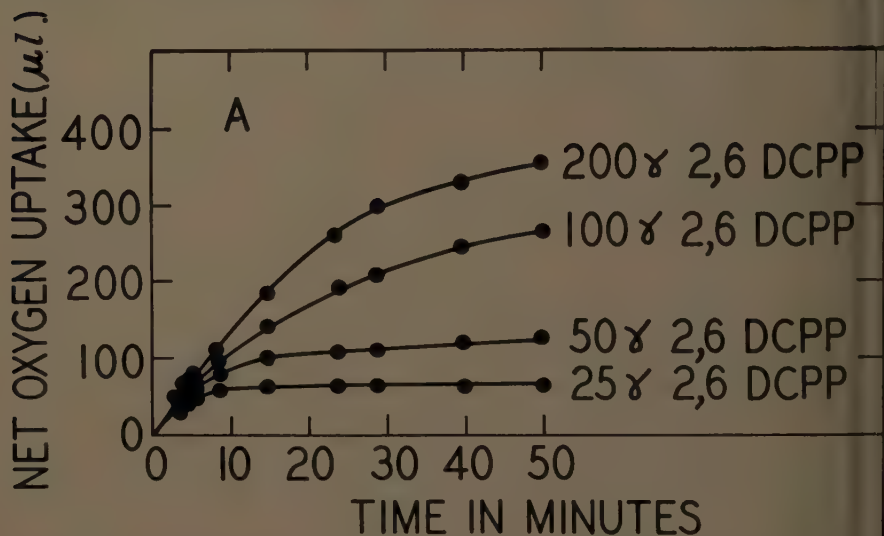


FIGURE 6. The effect of various amounts of reduced 2,6-dichlorophenolindophenol (2,6-DCPP) on *p*-hydroxyphenylpyruvate oxidation. Flasks contained 20 μ moles of *p*-hydroxyphenylpyruvic acid.¹⁵

It is obvious that the amount of substrate oxidized is proportional to the amount of dye present. Similar effects are observed with ascorbic acid in place of the dye, but much larger amounts of the vitamin are necessary to produce the same degree of protection, at least 150 times as much, on a molar basis.¹⁵

Effect of Ascorbic Acid on Tyrosine Metabolism in Vivo

With the *in vitro* demonstration that ascorbic acid protects *p*-hydroxyphenylpyruvic acid oxidase from inhibition by its substrate, it was of interest to see whether this inhibition also takes place *in vivo* in scorbutic guinea pigs and whether the same mechanism operates to explain the defect in tyrosine metabolism. If this were the case, the tyrosyluria would result from the induced enzymatic block, leading to the accumulation and excretion of *p*-hydroxyphenylpyruvic acid and related compounds.

Such *in vivo* studies require that there be a means of detecting inhibited *p*-hydroxyphenylpyruvic acid oxidase. A method to do so was suggested by our *in vitro* studies with reduced 2,6-dichlorophenolindophenol.¹⁵ The dye not only can prevent inhibition by excess substrate, but also has the ability to reactivate enzyme preparations previously inhibited by *p*-hydroxyphenylpyruvic acid unless the degree of inhibition is nearly complete.

The effect of the dye in the spectrophotometric assay of *p*-hydroxyphenylpyruvic acid oxidase activity is shown in FIGURE 7. The enzyme preparation in these experiments was a liver homogenate from a scorbutic guinea pig not treated with *p*-hydroxyphenylpyruvic acid. The activity of the *p*-hydroxyphenylpyruvic acid oxidase was measured by following the rate of disappearance of substrate spectrophotometrically.¹¹ The initial rate of substrate disappearance is the same with and without 2,6-dichlorophenolindophenol, although the initial rate is maintained for a longer time in the presence of the dye.

FIGURE 8 shows a similar assay, using a liver homogenate from a scorbutic guinea pig injected 1 hour prior to sacrifice with *p*-hydroxyphenylpyruvic acid intraperitoneally. It can be seen that the rate with dye is about the same as the rate with homogenate from the scorbutic animal not injected with *p*-hydroxyphenylpyruvic acid (FIGURE 7). Curve A is a much slower rate, indicating a partial inhibition of the oxidase, although the inhibited activity can be restored in part by $4 \times 10^{-3} M$ ascorbic acid (Curve B), and more effectively by 100 γ of reduced 2,6-dichlorophenolindophenol (Curve C).

TABLE 2 summarizes the data obtained in studies on the effect of injecting *p*-hydroxyphenylpyruvic acid intraperitoneally, 20 mg. per 100 gm. body weight, in scorbutic and nonscorbutic guinea pigs. One hour after the injection the animals were sacrificed and the liver enzymes assayed as indicated. No significant changes occurred in tyrosine transaminase and homogentisic acid oxidase under these conditions. *p*-Hydroxyphenylpyruvic acid oxidase activity was about the same in the normal and scorbutic animals not injected with *p*-hydroxyphenylpyruvic acid. However, the scorbutic animals injected with *p*-hydroxyphenylpyruvic acid had about one half of the activity of those not injected, and the inhibited enzyme activity could be restored by the addition of reduced 2,6-dichlorophenolindophenol. A similar inhibition of the oxidase was also evident after the intraperitoneal injection of phenylpyruvate to scorbutic guinea pigs, which was reversed by the addition of reduced dye. From these results, it was concluded that ascorbic acid acts *in vivo* as it does *in vitro*, that is, by protecting *p*-hydroxyphenylpyruvic acid oxidase from inhibition. More recently, these conclusions have been confirmed by feeding tyrosine to vitamin C-deficient guinea pigs in our laboratory³¹ and by Knox and Gosmi.³² Data illustrating the inhibition of liver *p*-hydroxyphenylpyruvic acid oxidase by feeding tyrosine is shown in TABLE 3. Normal and scorbutic guinea pigs were fed 400 mg. of tyrosine divided into 4 doses of 100 mg. at hourly intervals. One hour after the last dose of tyrosine, the animals were sacrificed and the tyrosine oxidation enzymes assayed. Normal animals showed no significant changes in the 3 enzymes assayed, but the scorbutic group had more than 80 per cent of *p*-hydroxyphenylpyruvic acid oxidase in liver and kidney. There was also a considerable increase in tyrosine transaminase activity in the

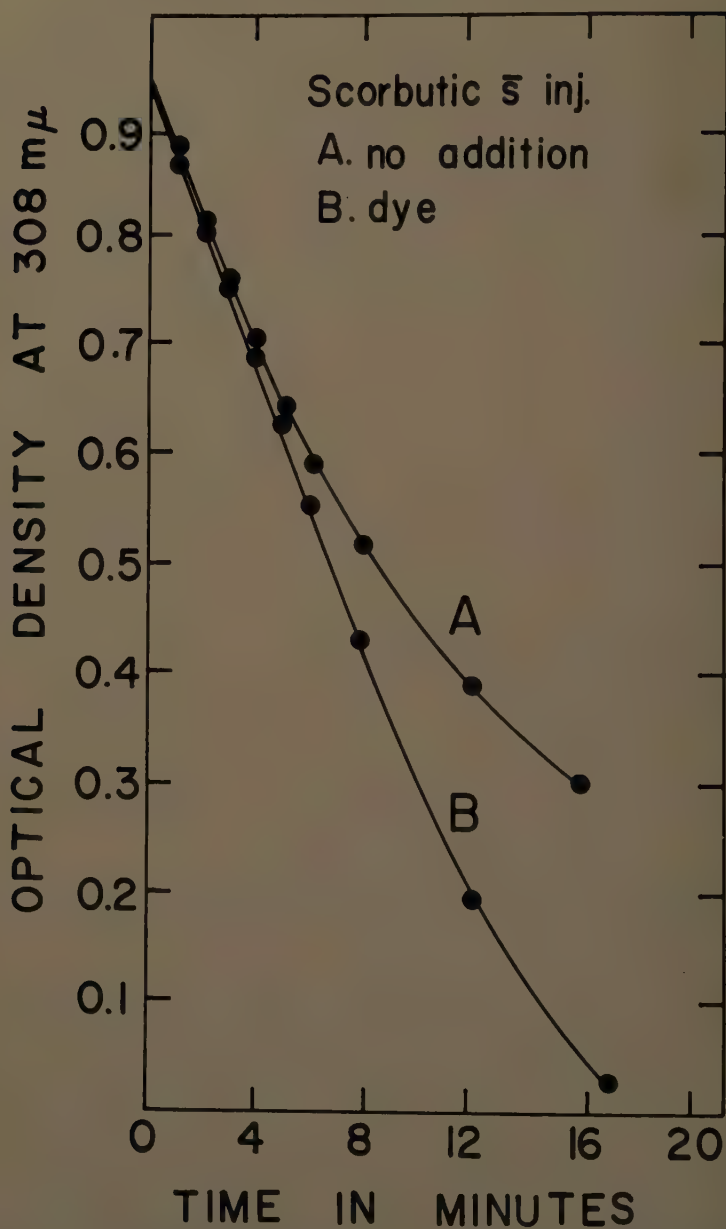


FIGURE 7. The spectrophotometric assay of *p*-hydroxyphenylpyruvic acid oxidase activity in liver homogenate from a scorbutic guinea pig not previously treated with *p*-hydroxyphenylpyruvic acid. Curve A, assay without dye; Curve B, assay with 2,6-dichlorophenolindophenol.³⁰ Reproduced by permission from *The Journal of Biological Chemistry*.

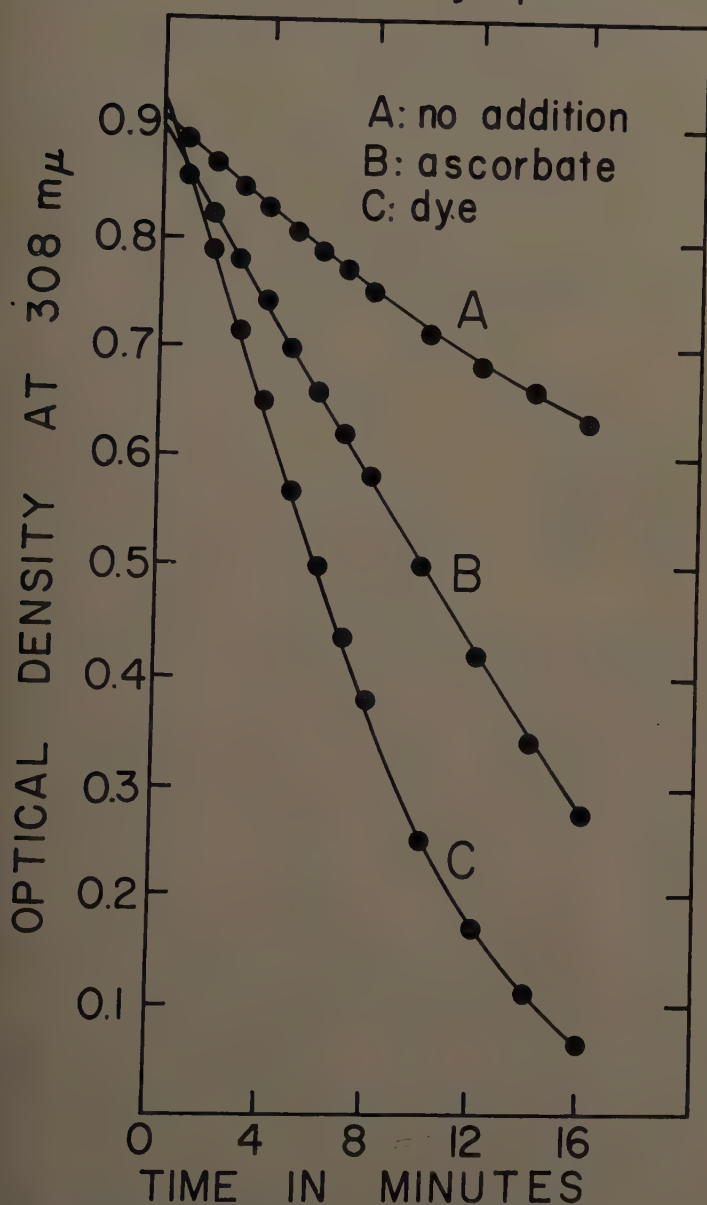
Scorbutic inj. \bar{c} pHPP

FIGURE 8. The spectrophotometric assay of *p*-hydroxyphenylpyruvic acid oxidase activity in liver homogenate from a scorbutic guinea pig injected intraperitoneally with *p*-hydroxyphenylpyruvic acid 1 hour before sacrifice.³⁰ Reproduced by permission from *The Journal of Biological Chemistry*.

scorbutic group, particularly after feeding tyrosine. The decrease in *p*-hydroxyphenylpyruvic acid oxidase and the increase in tyrosine transaminase would both contribute to an accumulation of *p*-hydroxyphenylpyruvic acid.

Urine collected during the 5-hour experimental period was analyzed for tyrosyl compounds, and these results are shown in TABLE 4. The scorbutic animals excreted about 40 mg. of *p*-hydroxyphenylpyruvic acid and a very small amount of *p*-hydroxyphenyllactic acid. No significant amount of tyrosine was found in the urine. The plasma levels of tyrosine at the time of sacrifice

TABLE 2
EFFECT OF INTRAPERITONEAL *P*-HYDROXYPHENYLPYRUVATE ON LIVER
ENZYMES IN NORMAL AND SCORBUTIC GUINEA PIGS

Enzymes	Normal		Scorbutic	
	Untreated (9)	Treated (9)	Untreated (8)	Treated (9)
	(μ M substrate oxidized/hr./gm. liver)			
Tyrosine transaminase	19	18	26	25
<i>p</i> HPP oxidase without 2,6-DCPP	33	29	27	13
<i>p</i> HPP oxidase with 2,6-DCPP	32	28	30	26
Homogentisic acid oxidase	93	100	75	77

See text for details. Numbers in parentheses indicate the number of animals in group.³⁰

TABLE 3
EFFECT OF FEEDING TYROSINE ON ENZYMES IN NORMAL AND SCORBUTIC GUINEA PIGS

Enzymes	Normal		Scorbutic	
	Untreated (6)	Treated (6)	Untreated (10)	Treated (10)
	(μ M substrate oxidized/hr./gm. tissue)			
<i>Liver</i>				
Tyrosine transaminase	18	24	40	87
<i>p</i> HPP oxidase	30	25	17	3
HGA oxidase	127	133	70	101
<i>Kidney</i>				
<i>p</i> HPP oxidase	7	5	7	1

See text for details.³¹

fice were elevated in both groups fed tyrosine, and were much higher in the scorbutic group, in agreement with the urinary findings. It is of interest that guinea pigs did not have to be frankly scorbutic to be susceptible to inhibition of *p*-hydroxyphenylpyruvic acid oxidase. In other experiments, guinea pigs placed on the vitamin C-free diet for only 1 week showed similar enzymatic and urinary changes when fed extra tyrosine.

In order to determine whether ascorbic acid is specifically required *in vivo* to prevent inhibition of *p*-hydroxyphenylpyruvic acid oxidase, several other reducing agents were tested in place of L-ascorbic acid. Groups of guinea pigs were placed on the vitamin C-free diet for 1 week and then given 400 mg. of tyrosine in 100-mg. portions at hourly intervals, as before. The reducing

agents were given intraperitoneally, 10 mg. one-half hour before the first tyrosine feeding and 10 mg. more 2 hours later. Animals were sacrificed 1 hour after the last feeding of tyrosine, and the *p*-hydroxyphenylpyruvic acid oxidase activity of the liver was determined. Plasma levels of tyrosine, *p*-hydroxyphenylpyruvic acid, and liver ascorbic acid were also measured at the time of sacrifice (TABLE 5). The results indicate that 2,6-dichlorophenolindophenol and D-isoascorbic acid were able to prevent inhibition of *p*-hydroxyphenylpyruvic acid oxidase. In addition, D-glucoscorbic acid gave partial protection

TABLE 4

PLASMA TYROSINE AFTER FEEDING TYROSINE TO NORMAL AND SCORBUTIC GUINEA PIGS

Plasma tyrosine (mg. %)	Normal		Scorbutic	
	Untreated	Treated	Untreated	Treated
	0.5	12	1.0	73
tyrosine				
<i>p</i> HPP (mg. excreted in 5 hours)	<0.1	0.6	<0.1	38
<i>p</i> HPL (mg. excreted in 5 hours)	<0.1	<0.1	<0.1	0.4

See text for details.³¹

TABLE 5

PREVENTION OF *p*-HYDROXYPHENYLPIYRUVIC ACID OXIDASE
INHIBITION *IN VIVO* BY VARIOUS COMPOUNDS*

Compound†	Liver <i>p</i> HPP oxidase (μ M/hr./gm.)	Plasma levels		Liver "ascorbic acid" (mg. %)
		L-Tyrosine (mg. %)	<i>p</i> HPP (mg. %)	
tyrosine (6)	1.6	50.3	5.6	3.1
Ascorbic (4)	32.2	12.5	<0.4	28.9
isoascorbic (4)	31.0	13.2	<0.1	13.3
Glucoscorbic (4)	9.5	24.5	0.8	4.5
5-DCPP, ox. (5)	29.0	10.2	<0.1	2.6
5-DCPP, red. (4)	29.5	5.9	<0.1	3.3

* Modified from data of Zannoni and La Du's TABLE 4.³¹

† Ten mg. injected I.P. 30 min. before first tyrosine feeding and 10 mg. again 2 hours later.

the enzyme. The concentration of tyrosine in the plasma was elevated in the groups with inhibited *p*-hydroxyphenylpyruvic acid oxidase and was proportional to the degree of inhibition observed. The concentration of apparent ascorbic acid in the liver indicated that there was a greater retention of D-isoascorbic acid than D-glucoscorbic acid; this finding was in agreement with their relative ability to protect *p*-hydroxyphenylpyruvic acid oxidase. All of these compounds were previously shown to protect *p*-hydroxyphenylpyruvic acid oxidase *in vitro* from inhibition by excess substrate. The fact that several of these compounds are also effective *in vivo* is further evidence that L-ascorbic acid is not specifically required to maintain tyrosine metabolism. Further

TABLE 6
PREVENTION OF pHPP OXIDASE INHIBITION WITH FOLIC ACID*
IN SCORBUTIC GUINEA PIGS FED EXTRA TYROSINE

Folic acid (mg.)	No. animals	Tyrosine transam.	pHPP oxidase	Plasma tyrosine (mg. %)	Urine pHPP (mg.)	Liver "ascorbic acid" (mg. %)
		(μM/hr./gm. liver)				
—	6	40	3.8	48.3	38.3	1.6
20	7	47	32.9	4.3	0.1	3.3
2	10	36	13.0	2.9	1.1	2.3

* Folic acid injected I.P. prior and during the oral administration of L-tyrosine
See text for details.

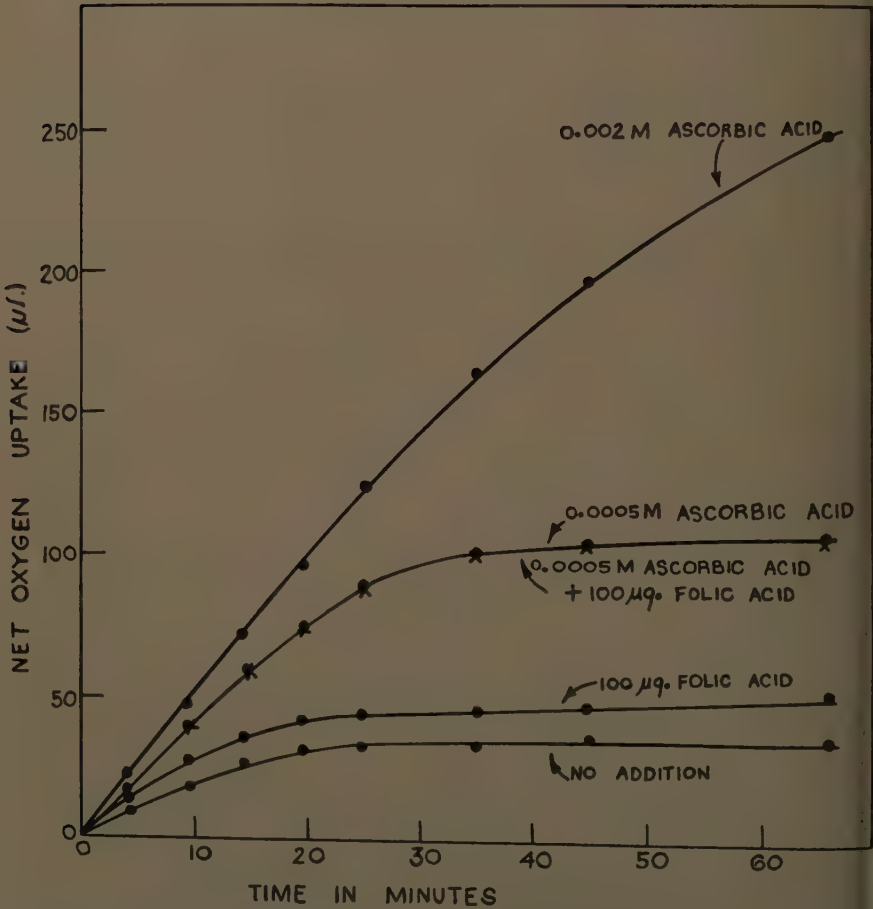


FIGURE 9. Effect of folic acid alone and in combination with ascorbic acid on the oxidation of 10 μmoles of tyrosine with liver homogenate from a folic acid-deficient guinea pig.

evaluation of the antiscorbutic activity of 2,6-dichlorophenolindophenol has shown that this reducing agent does not protect guinea pigs from developing weight loss, hemorrhages, and other signs of scurvy. Failure of the dye to replace ascorbic acid in the formation and maintenance of dental structures are discussed by Harold M. Fullmer *et al.* elsewhere in these pages.

Another compound found to protect the oxidase from inhibition under the above experimental conditions was folic acid (TABLE 6). The intraperitoneal injection of 20 mg. of folic acid gave as much protection in scorbutic guinea pigs as was given by ascorbic acid. However, 2 mg. of folic acid gave only partial protection, approximately 50 per cent, and smaller amounts were even less effective. The "antiscorbutic" activity of folic acid observed here is in agreement with the results of Woodruff and Darby⁶ and supports the possibility that folic acid is involved in tyrosine metabolism, even though the amount of folic acid required to show any effect is high.

Further studies were undertaken on the effect of folic acid in this reaction. The adequacy of folic acid in the scorbutic diet previously used was assured by supplementing the diet with extra folic acid orally and by intraperitoneal injection. The scorbutic animals on this diet were still susceptible to inhibition of *p*-hydroxyphenylpyruvic acid oxidase by tyrosine feeding unless large amounts of extra folic acid or ascorbic acid were given.

Guinea pigs were also placed on a folic acid-deficient diet for 4 weeks. Folic acid-deficient guinea pigs were not found to be susceptible to inhibition by tyrosine feeding, nor was any tyrosyluria observed.

Liver homogenates prepared from folic acid-deficient guinea pigs were also used to study tyrosine oxidation *in vitro* (FIGURE 9). It is apparent that the oxidation of tyrosine in such preparations was not influenced by the addition of folic acid alone or in combination with a suboptimal amount of ascorbic acid.

It must be concluded, therefore, that folic acid acts *in vivo* only at very high concentrations and has no direct effect *in vitro* on tyrosine oxidation. Folinic acid and prefolic acid, another reduced form of folic acid kindly supplied to us by John C. Keresztesy of our institute, were also ineffective.

Conclusions

In conclusion, the role of ascorbic acid in tyrosine metabolism has been shown to be a rather unusual one for a vitamin. Instead of acting as a cofactor for a particular enzymatic step, that is, as members of the B family of vitamins, ascorbic acid has the ability to protect an enzyme, *p*-hydroxyphenylpyruvic acid oxidase, from inhibition by its substrate.

It should be emphasized that this protection is necessary only under unusual conditions, when large amounts of tyrosine are being metabolized. It does not seem to be necessary to maintain normal tyrosine oxidation under ordinary dietary conditions.

A clear distinction between the effects of vitamin C on tyrosine metabolism and its antiscorbutic properties is emphasized by the ability of 2,6-dichlorophenolindophenol completely to replace the vitamin as far as maintaining tyrosine oxidation and yet be devoid of any antiscorbutic activity.

Perhaps future experiments will reveal instances in which ascorbic acid acts

as a "conventional" vitamin in some of the other biochemical processes that are deranged in scurvy. The elucidation of its role in tyrosine metabolism illustrates one of the variety of ways in which this essential carbohydrate participates in biochemical and physiological reactions.

Acknowledgments

We acknowledge the participation of George Jacoby and Stephen Malawista in the studies on the effects of folic acid carried out in our laboratory. We are indebted to Mary E. Reid of the Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, for her advice and help in preparing the diet used in these studies.

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ASCORBIC ACID IN TYROSINE METABOLISM

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A period in ascorbic acid research has now ended with agreement that the defect in tyrosine metabolism is not caused by ascorbic acid deficiency. It is caused by the high dose of tyrosine used to demonstrate the defect, which super-venes only when the body is less than saturated with ascorbic acid. These results deserve a historical review not only for their own value but also because of a new direction they have given to research in tyrosine metabolism. There may be, after all, something in common among the actions of ascorbic acid in the different hydroxylating enzyme systems.

Even before we demonstrated the need for ascorbic acid in the oxidation of *p*-hydroxyphenylpyruvate (*p*HPP) by liver homogenates (Knox and LeMay-Knox, 1951), Painter and Zilva (1947) had come to the conclusion that the accumulation and excretion of this compound by scorbutic guinea pigs fed tyrosine was not a specific manifestation of scurvy. They did not find *p*HPP excretion by scorbutic guinea pigs unless unphysiologically high doses of tyrosine were given. With such doses of tyrosine, *p*HPP was excreted by guinea pigs long before they were scorbutic. Human studies later confirmed these findings. Nevertheless, we were concerned with the logical possibility that the low amounts of tyrosine normally degraded also required the low antiscorbutic amounts of ascorbic acid, although the traces of accumulated *p*HPP could not be demonstrated in the unsupplemented scorbutic guinea pig. Direct demonstration of an enzymic defect in *p*HPP oxidation by extracts of liver from scorbutic animals was precluded until recently. Either ascorbic acid or one of the other activators that does the same thing had to be added to both normal and scorbutic enzyme preparations for them to be active. With this addition there was no abnormality of *p*HPP oxidation in liver extracts from scorbutic animals.

The new spectrophotometric assay of *p*HPP oxidase (Lin *et al.*, 1958; Hager *et al.*, 1957) did not require addition of ascorbic acid or other activators. With this assay it was demonstrated directly that the *p*HPP oxidase system was intact in the extracts of liver from scorbutic guinea pigs (Knox, 1959). This fully proved the contention of Painter and Zilva. The remaining possibility was that the tyrosine dosage produced the enzymic defect, and this was demonstrated (Knox and Goswami, 1960; La Du and Zannoni, 1960). The metabolic defect produced by tyrosine dosage is actually an enzymic imbalance, consisting of an adaptive increase in the activity of tyrosine transaminase which forms *p*HPP, and a reversible inactivation of *p*HPP oxidase that removes *p*HPP (FIGURE 1). Therefore *p*HPP accumulates and is excreted unless sufficient ascorbic acid is available to prevent the inactivation of *p*HPP oxidase.

The inactivation of *p*HPP oxidase can be produced *in vivo* and *in vitro*, but the inactivation can be prevented in both situations by ascorbic acid or dichlorophenolindophenol; it can also be reversed by a preliminary incubation of the enzyme with one of these activators plus glutathione. In our laboratory simple addition of the activators to the reaction mixture does not reverse the inactivation.

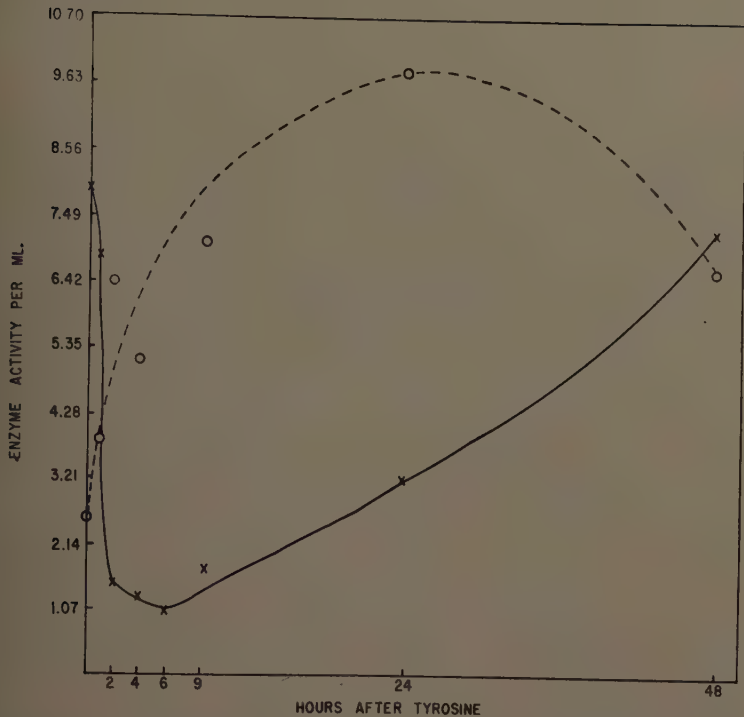


FIGURE 1. Time sequence of *in vivo* activity changes of liver tyrosine- α -ketoglutarate aminase (—○—) and pHPH oxidase (—×—) following an oral dose of 2.7 mmoles tyrosine to normal guinea pigs. Enzyme activities are $\Delta \mu$ moles of pHPH per hour per milliliter of 25 per cent liver homogenate. Each point is the average from two animals.

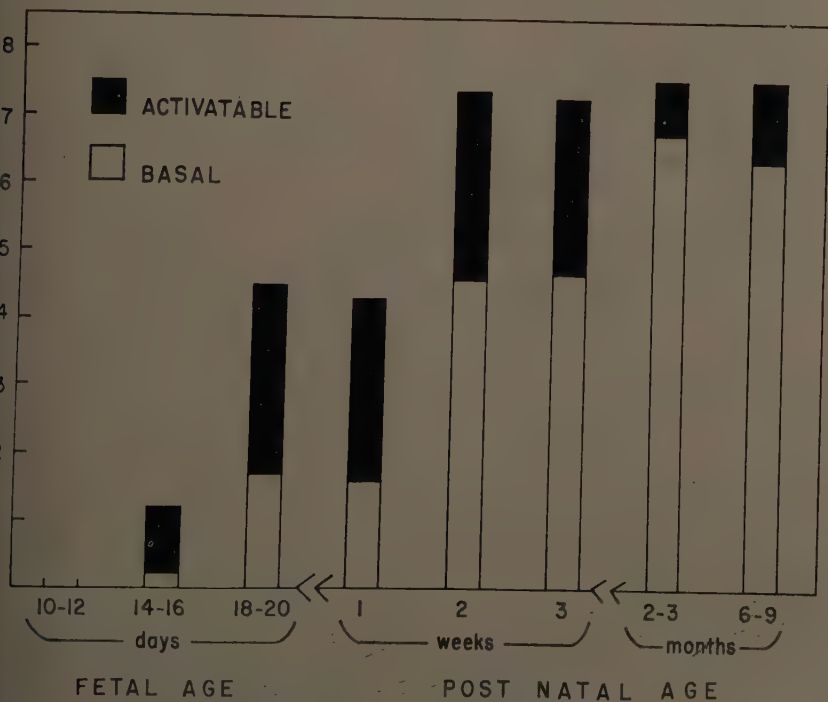


FIGURE 2. Effect of age on basal and activatable pHPH oxidase activity of rat liver.

vation. The chemical nature of the inactivated form intrigued us even more when we found it occurred as a normal stage in the development of *p*HPP oxidase in the livers of fetal and newborn rats (FIGURE 2). The enzyme appears late in gestation; while it develops to the normal adult level, part of this enzyme is an "activatable" form with properties identical to that of the inactivated enzyme from tyrosine-fed guinea pigs (Goswami and Knox, unpublished). The point of interest in *p*HPP oxidation has thus shifted to the chemical mechanism of its reaction and of its reaction-inactivation. Ascorbic acid or other activators of this mechanism may play a physiological role here, although the effect is not a part of scurvy.

Tyrosine metabolism, therefore, is still related to the problem of ascorbic acid action in several ways. The tyrosine-induced defect in tyrosine metabolism is prevented by higher-than-antiscorbutic amounts of ascorbic acid, and this remains one of the few pieces of evidence to recommend such higher intakes. The physiological occurrence of the inactive form of the enzyme during development points to a role such as that of ascorbic acid in the genesis of the active enzyme. Finally, the enzyme hydroxylates an aromatic ring, a relatively new type of reaction that has also been associated with ascorbic acid in connection with the formation of hydroxyproline, 5-hydroxytryptophan, and epinephrine.

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ROLE OF ASCORBIC ACID IN MICROSOMAL ELECTRON TRANSPORT AND THE POSSIBLE RELATIONSHIP TO HYDROXYLATION REACTIONS

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In microsomes of pig and beef adrenals, an ascorbic acid-dependent DPNH* oxidase has been demonstrated (Kersten *et al.*^{1,2}). FIGURE 1 shows the basic experiment: DPNH is not, or only to a very small extent, oxidized by the adrenal microsomes alone; after addition of ascorbic acid, a marked DPNH oxidation can be seen.

Principally the same phenomenon—an ascorbic acid-dependent DPNH oxidation—has been demonstrated in rat liver and kidney microsomes.³ In contrast to the adrenal microsomes, the liver and kidney microsomes have a high activity in an ascorbic acid-independent DPNH oxidation. This fact is now easily understood by the finding of Strittmatter *et al.*,⁴ who demonstrated a flavoprotein-catalyzed electron transport mechanism: DPNH → cytochrome b_5 in liver microsomes. Cytochrome b_5 itself is autoxidizable. The microsomal ascorbic acid-dependent DPNH oxidase is strictly specific for DPNH. Under the conditions of these experiments, TPNH is not oxidized (FIGURE 2).

The ascorbic acid-dependent DPNH oxidation is not inhibited by cyanide. Fe^{++} and *p*-chloromercuribenzoate are strong inhibitors. The specificity of the enzyme for ascorbic acid is not as high as for the nucleotide; for example, isoascorbic acid also has some but a distinctly lower activity (FIGURE 2). The ascorbic acid, however, cannot be replaced by dehydroascorbic acid.^{1,2,5}

The role of the ascorbic acid in the microsomal DPNH oxidation can also be demonstrated by manometric measurements of the oxygen consumption in the Warburg apparatus.⁶ FIGURE 3 shows that only in the presence of catalytic amounts of ascorbic acid a distinct oxygen consumption is observed; this amount corresponds fairly well with the theoretical amount calculated for complete oxidation of the DPNH present in the vessel. So it is evident that there is actually an electron transport from the DPNH to oxygen. What is the function of the ascorbic acid in this electron transport chain? As already shown in FIGURE 2, dehydroascorbic acid cannot act as an intermediate hydrogen acceptor. On the other hand, it is entirely impossible for ascorbic acid to act as an intermediate hydrogen acceptor in its reduced form. We therefore postulated a “radical-like semioxidized” monodehydroascorbic acid that lost only one hydrogen, that is, one electron.^{1,2,5}

The same conclusion was drawn at about the same time by Kern *et al.*⁷ and Nason *et al.*⁸ when these two teams also described a plant DPNH oxidase that was ascorbic acid-dependent. Meanwhile clear evidence for the reality of such a radical-like monodehydroascorbic acid has been brought up by differ-

The following abbreviations are used: DPN = diphosphopyridine nucleotide; DPNH = reduced diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide; TPNH = reduced triphosphopyridine nucleotide.

ent methods,⁹ recently even by direct measurement by electron resonance spectroscopy (Yamazaki *et al.*¹⁰).

The existence of such a transitory oxidized form of the ascorbic acid acting as electron carrier between reduced nucleotide and oxygen includes the postulate for an ascorbic acid-oxidizing factor or enzyme. By such an enzyme the steady state concentration of the "acceptor form" of the ascorbic acid, that is, of the monodehydroascorbic acid, can be enhanced (FIGURE 4).

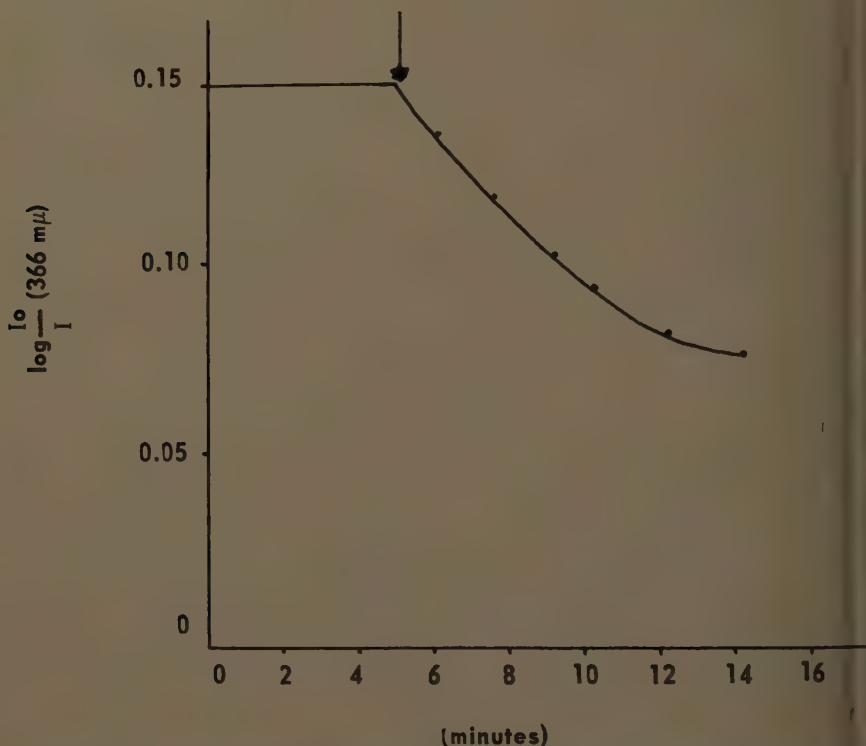


FIGURE 1. DPNH oxidation by adrenal microsomes (optical test): 0.06 *M* phosphate buffer; pH 7.4; 37° C.; 4.5×10^{-5} *M* DPNH; microsomes, 0.5 mg. protein 2.5 ml. (biuret); 2.3×10^{-3} *M* ascorbic acid.

In this experiment, cucumber ascorbic acid oxidase* was used.¹¹ FIGURE 1 shows that under the condition of the optical test the oxidation of the ascorbic acid is the rate-limiting step. When this step was accelerated by adding terminal oxidase, the whole reaction was activated. The ascorbic acid concentration could be reduced to one- or two-tenth power without a remarkable change in the reaction speed. This means that the enzyme was saturated with the acceptor form, that is, the monodehydroascorbic acid. Under those conditions catalytic amounts of ascorbic acid were already effective. In the

* We are indebted to R. Dawson, New York, N.Y., for a sample of pure ascorbic acid oxidase. Our own preparations have been checked with this original pure oxidase.

st with ascorbic acid oxidase present, the rate-limiting step was the enzymatic hydrogen transport from DPNH to the monodehydroascorbic acid. If PNH was present in excess, that is, enzyme-saturated (FIGURE 5), the rate reaction depended only on the concentration of microsomal protein (Sack, H. J. Staudinger, 1960, unpublished) (FIGURE 5).

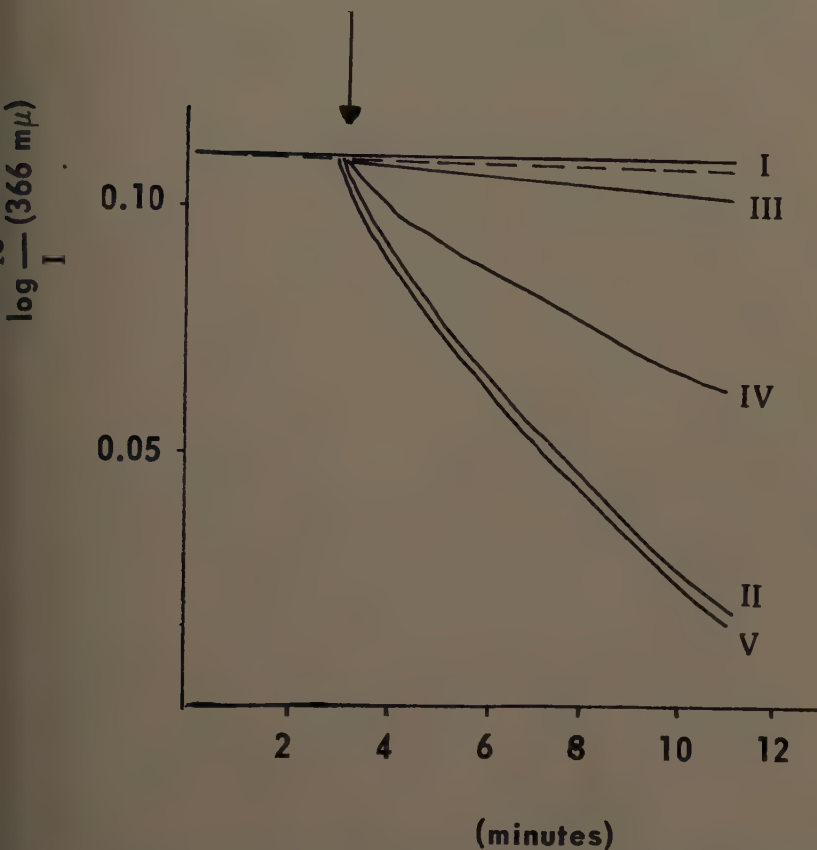


FIGURE 2. Specificity of the ascorbic acid-dependent DPNH oxidase. Medium and concentrations as in FIGURE 1 except: (I) $3.2 \times 10^{-3} M$ TPNH, $2.3 \times 10^{-3} M$ ascorbic acid; (II) $3.2 \times 10^{-5} M$ DPNH, $2.3 \times 10^{-3} M$ ascorbic acid; (III) $2.3 \times 10^{-3} M$ dehydroascorbic acid; (IV) $2.3 \times 10^{-3} M$ D-isoascorbic acid; (V) $2.3 \times 10^{-3} M$ ascorbic acid + $1 \times 10^{-3} M$ KCN.

the enzyme is transporting the hydrogen or the electron from DPNH to monodehydroascorbic acid; therefore, according to the nomenclature of Mann-Ostenhoff,¹² we called it DPNH-monodehydroascorbic acid-transhydrogenase. It seems to be a flavoprotein, and SH groups are essential, as the inhibition with *p*-chloromercuribenzoate indicated.^{1,2} The Michaelis constant is of the order $10^{-5} M$.^{1,2} We have not yet succeeded in isolating the enzyme from the microsomes as a pure protein.

As shown in FIGURE 3 the terminal electron acceptor for the ascorbic acid-dependent DPNH oxidase in microsomes is oxygen. Ascorbic acid must be oxidized by an oxidase in order to act as an intermediate electron transport metabolite. Then the question arose as to what the normal or "physiological" terminal ascorbic acid oxidase in animal microsomes could be. The DPNH

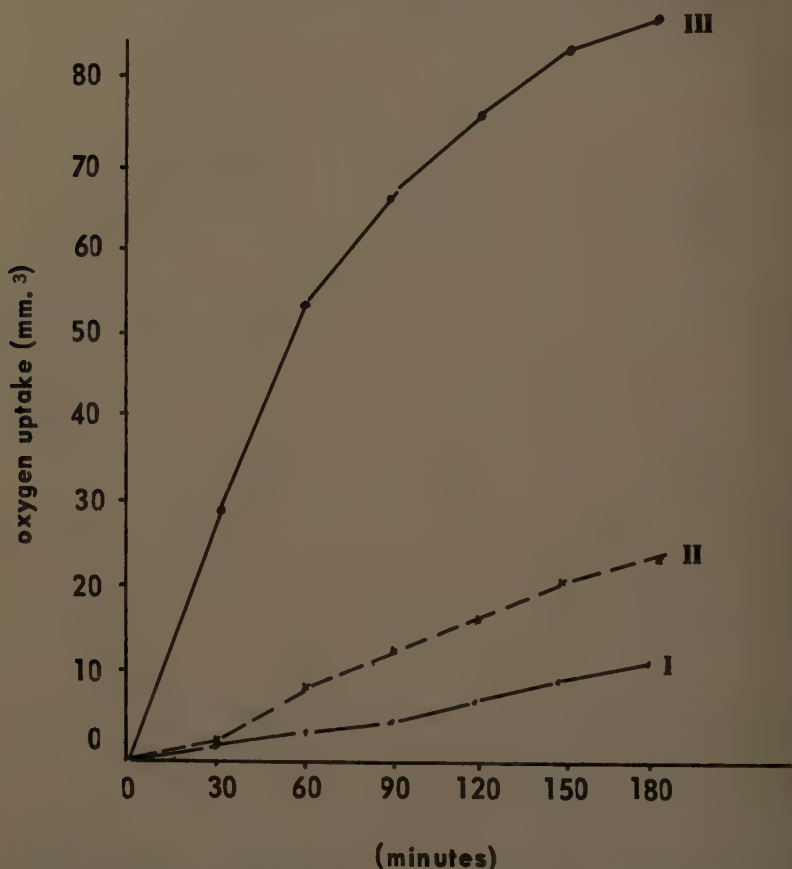


FIGURE 3. Oxidation of DPNH by adrenal microsomes (manometric measurement) 0.06 *M* phosphate buffer; 1×10^{-4} *M* Versene; pH 7.4; 37° C.; osc.: 104 per minute; volume 2.8 ml.; adrenal microsomes: 1.76 mg. protein (biuret) per vessel (2.8 ml.). (I) Ascorbic acid, 6×10^{-4} *M*; (II) 3×10^{-3} *M* DPNH; (III) 3×10^{-3} *M* DPNH, 6×10^{-4} *M* ascorbic acid.

oxidation by adrenal microsomes is insensitive against cyanide. So the assumption that microsomal cytochrome b_5 might act as the natural ascorbic acid oxidase in microsomes was not too hazardous. We have been able to isolate pure cytochrome b_5 from pig adrenal microsomes (Krisch *et al.*¹³). Evidence for this thesis was given by the experiments in the Warburg apparatus.^{6*}

As FIGURE 6 shows, cytochrome b_5 has a very distinct activation effect on

* For these experiments we isolated the cytochrome b_5 from hog liver microsomes following the method of Strittmatter *et al.*¹⁴ and Krisch *et al.*¹³

the oxygen consumption in these experiments with DPNH as substrate and adrenal microsomes as enzyme. This effect is again enhanced by catalytic amounts of ascorbic acid. It is insensitive against cyanide as must be expected since the autoxidation of cytochrome b_5 is also cyanide insensitive (FIGURE 7).

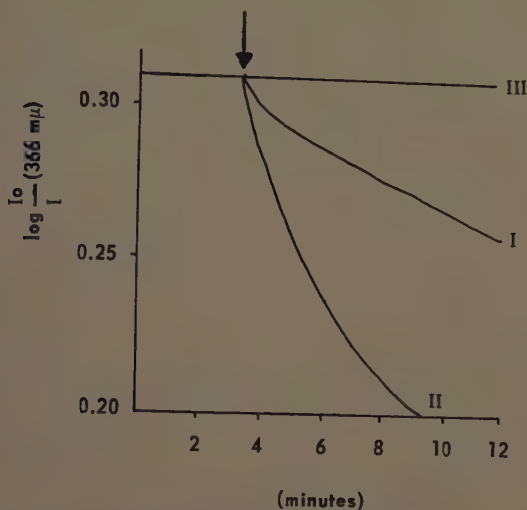


FIGURE 4. Effect of ascorbic acid oxidase on the DPNH oxidation by adrenal microsomes (optical test): 0.06 M phosphate buffer; pH 7.4; $37^\circ C$.; microsomes: 1.5 mg. protein 5 ml. (biuret); $1 \times 10^{-4} M$ DPNH. (I) Ascorbic acid, $1 \times 10^{-4} M$; (II) $1 \times 10^{-5} M$ ascorbic acid + 0.5 E per ml. ascorbic acid oxidase; (III) 0.5 E/2.5 ml. ascorbic acid oxidase

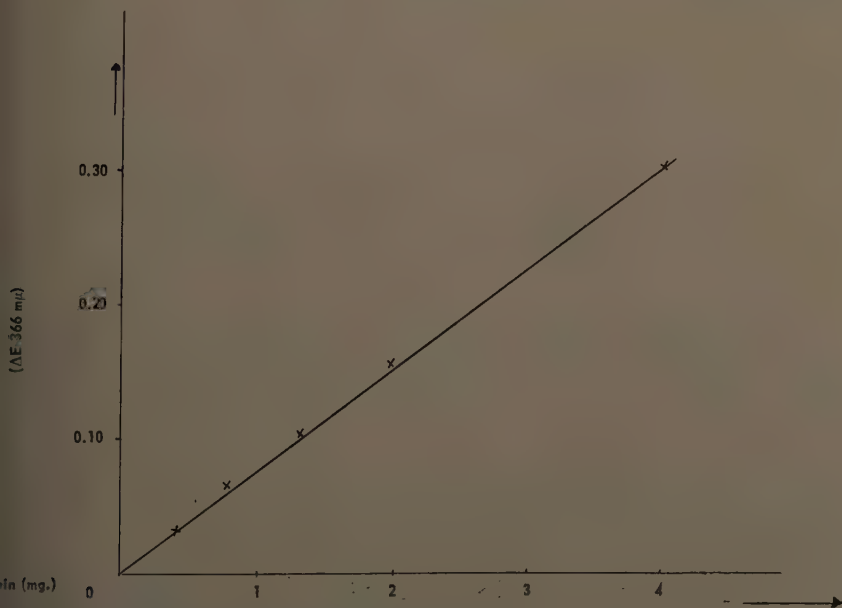


FIGURE 5. DPNH decrease as a function of enzyme concentration. Medium and concentrations as in FIGURE 4; volume 2.5 ml.; time 5 min.

A further proof for our view of the role of ascorbic acid in the microsomal electron transport is given in FIGURES 8 and 9.⁶ Ascorbic acid oxidase, as well as cytochrome b_5 , oxidizes ascorbic acid to dehydroascorbic acid,⁶ as is well known. By determination of the reduced ascorbic acid, the decrease can

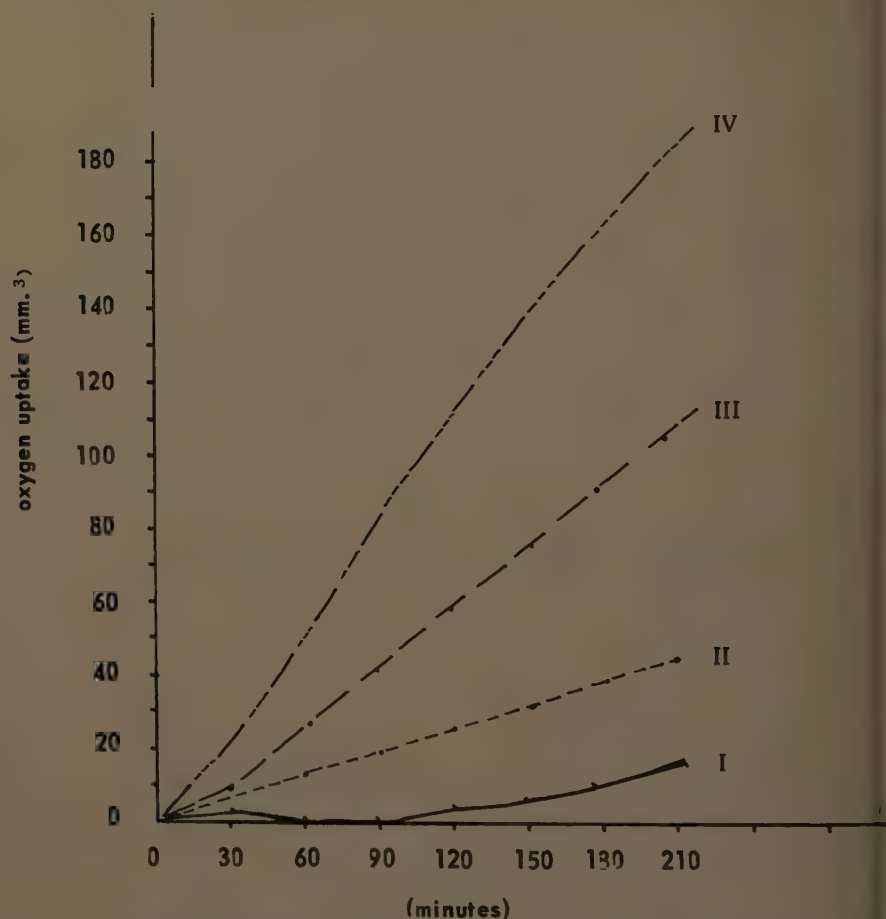
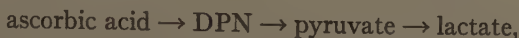


FIGURE 6. Influence of cytochrome b_5 and ascorbic acid on DPNH oxidation by adrenal microsomes (manometric measurements). Medium as in FIGURE 3; microsomes, 1.06 mg. protein/vessel (2.8 ml.); (I) DPNH, $5 \times 10^{-3} M$; (II) $5 \times 10^{-3} M$ DPNH + $5 \times 10^{-4} M$ ascorbic acid; (III) $5 \times 10^{-3} M$ DPNH + $5 \times 10^{-5} M$ cytochrome b_5 ; (IV) $5 \times 10^{-3} M$ DPNH + $5 \times 10^{-4} M$ ascorbic acid + $5 \times 10^{-5} M$ cytochrome b_5 .

be demonstrated (white bars of FIGURES 8 and 9). In the same system when DPNH was present, reduced ascorbic acid did not decrease (black bars). In other words, the first oxidation product, monodehydroascorbic acid, is promptly reduced by the DPNH. If the microsomal enzyme is omitted, the ascorbic acid will be oxidized even if DPNH is present.⁶

Summarizing all results, the scheme presented in FIGURE 10 for the ascorbic acid-dependent electron transport mechanism in microsomes can be proposed.

It was not possible to measure the standard potential (E_0') of the ascorbic acid \leftrightarrow monodehydroascorbic acid system. All attempts to measure it by the combined "back reaction,"



failed because of the insensitivity of the analytical procedure for the determina-

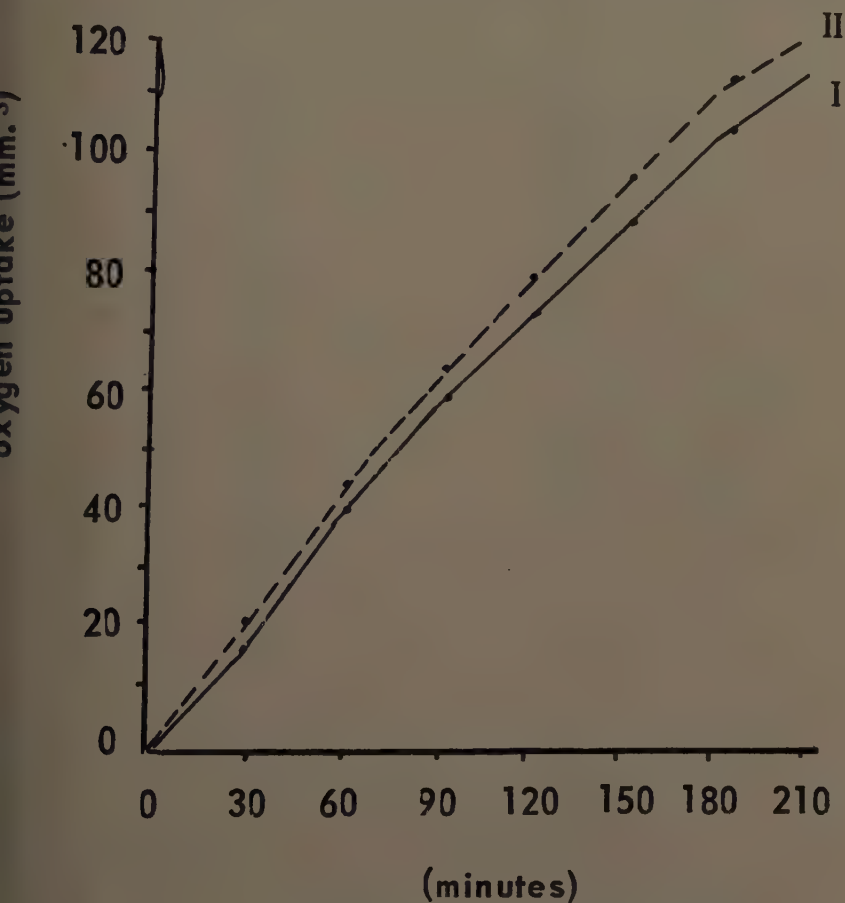


FIGURE 7. Influence of cyanide on cytochrome b_5 . Medium and concentrations as in FIGURE 6 except: (I) $2.5 \times 10^{-5} M$ cytochrome b_5 ; (II) $2.5 \times 10^{-5} M$ cytochrome b_5 + $4.6 \times 10^{-4} M$ cyanide.

on of such small amounts of lactate. Nevertheless an approximative value can be roughly estimated: it can not be more negative than zero volt since lactate would then be formed in detectable amounts; on the other hand, it must be more negative than +0.2 volt, which is the standard potential E_0' of the system: ascorbic acid \leftrightarrow dehydroascorbic acid. The fact that cytochrome b_5 , with a standard potential E_0' of +0.02 volt,¹⁵ is reduced by ascorbic acid^{6,13} does not help very much since, on the one hand, the concentration of the radi-

cal-like monodehydroascorbic acid is not known and, on the other hand, a steady state of the reaction is hardly likely to obtain because of the quick auto-oxidation of the cytochrome b_5 .

The role of the ascorbic acid in microsomal electron transport seems to be fairly well established, but the biological significance of this electron transfer mechanism in microsomes is not yet clear. As suggested in FIGURE 10, w

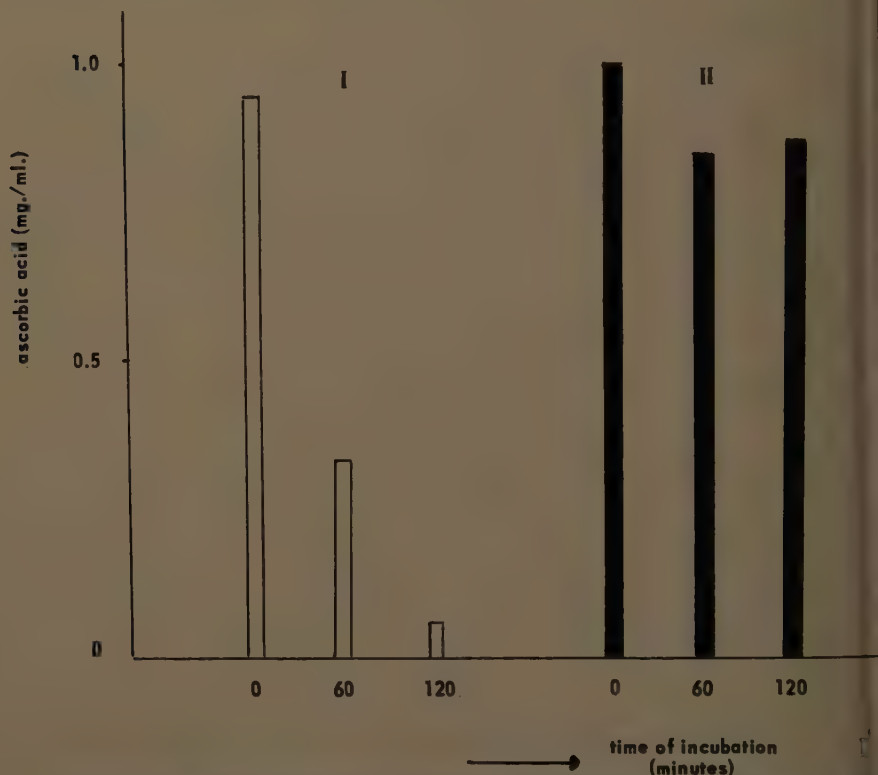


FIGURE 8. Oxidation of ascorbic acid by ascorbic acid oxidase with and without DPNH. Phosphate buffer, 0.06 M ; pH 7.4; $0.6 \times 10^{-2} M = 1.06$ mg./ml. ascorbic acid; adrenal microsomes, 15.2 mg. protein/vessel; volume, 2.8 ml.; $37^\circ C$; Warburg vessel, 104 osc./min. and (I) 0.5 E ascorbic acid oxidase (white bars); and (II) 0.5 E ascorbic acid oxidase + $3 \times 10^{-3} M$ DPNH (black bars).

presume that the first product of the reaction with oxygen is a kind of a hydroxy radical that may be used for hydroxylation reactions.^{1,2,3,17} Several years ago we published results indicating that ascorbic acid was involved in the biogenesis of corticosteroids in adrenal homogenates (Hofmann and Staudinger;¹⁶ Staudinger;³ Kersten *et al.*¹⁷) and that the rate of 11β -hydroxylation of deoxycorticosterone was increased by ascorbic acid (Kahnt and Wettstein;¹⁸ Kersten *et al.*¹⁷). All these results are known. We could not further elucidate the mechanism of these effects of ascorbic acid, partly because

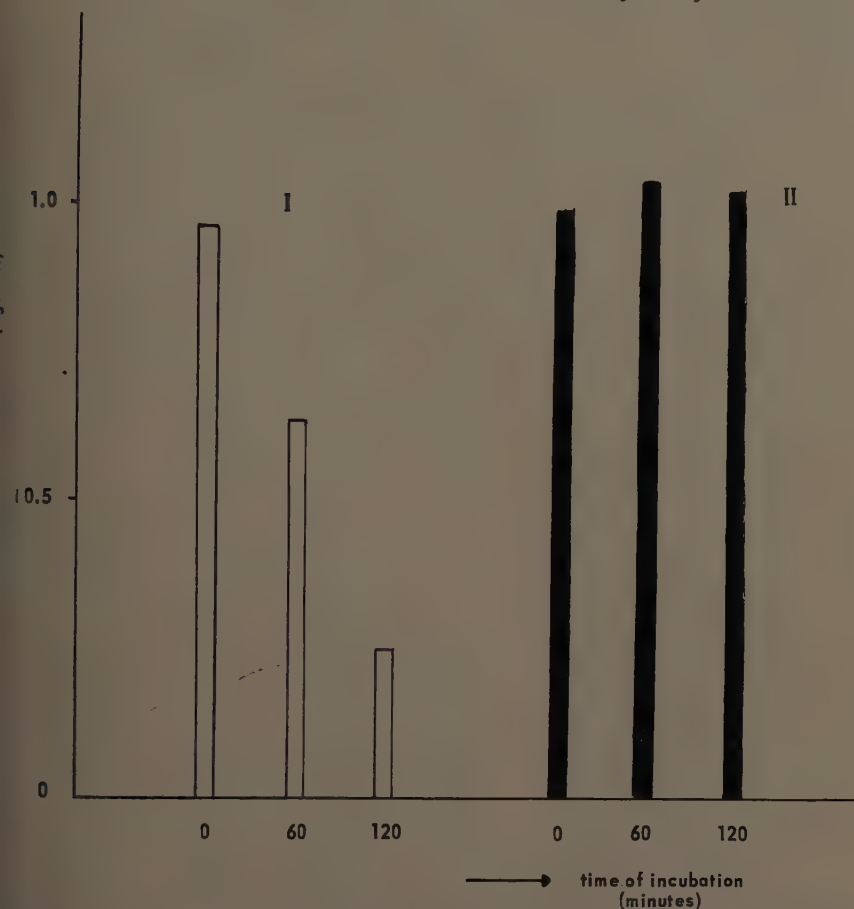


FIGURE 9. Oxidation of ascorbic acid by cytochrome b_5 with and without DPNH. Medium and concentrations as in FIGURE 8 except: (I, white bars) $5 \times 10^{-4} M$ cytochrome b_5 ; (II, black bars) $3 \times 10^{-2} M$ DPNH + $5 \times 10^{-4} M$ cytochrome b_5 .

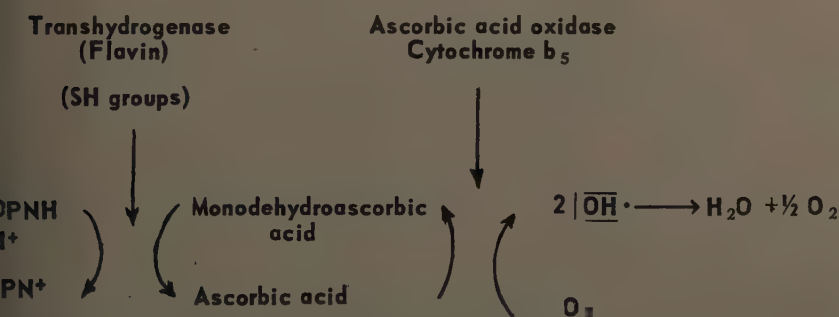


FIGURE 10. Microsomal electron transport chain.

of the complicated multienzyme system required for the different steps steroid biogenesis. Earlier and more recent results indicated, however, that the effect of ascorbic acid depends on different conditions, such as pH ,¹⁹ time of incubation, cyanide concentration, and the interval between the death of the

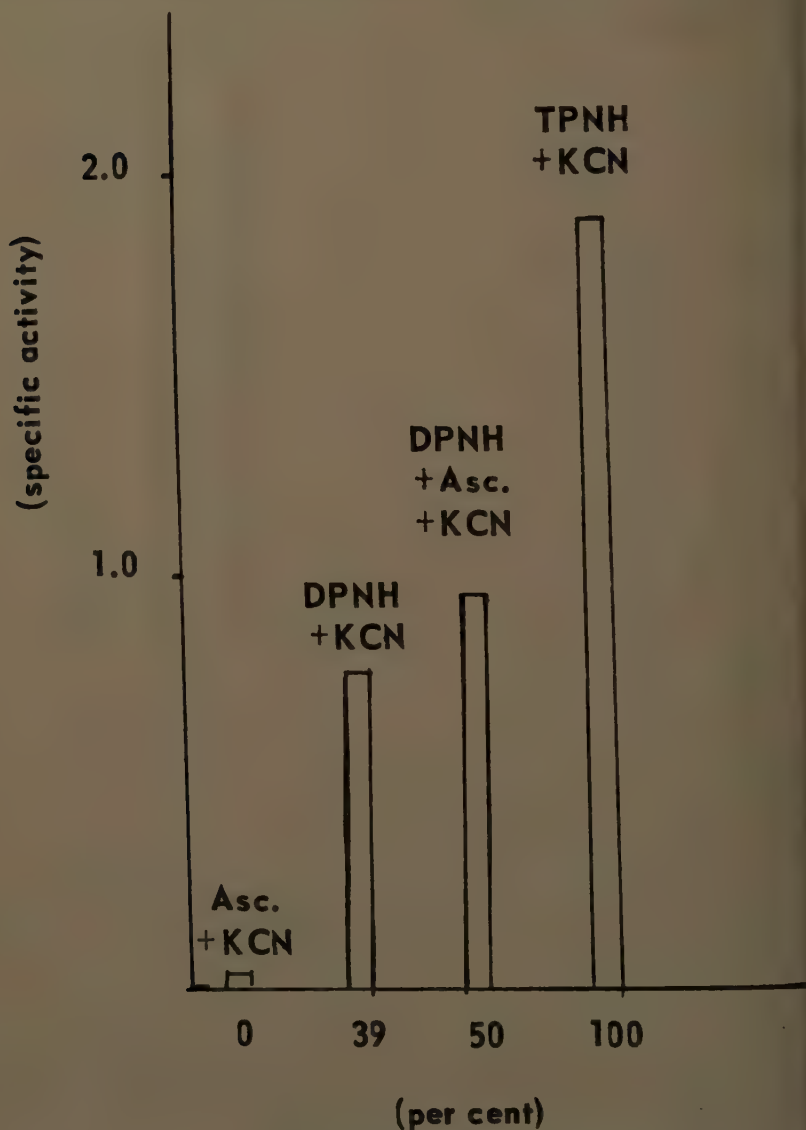


FIGURE 11. Influence of DPNH and ascorbic acid on acetanilide hydroxylation. Tris phosphate buffer 0.1 M ; pH 7.4; $2.0 \times 10^{-3} M$ TPNH; $2.0 \times 10^{-3} M$ DPNH; $2.0 \times 10^{-3} M$ ascorbic acid; $1.0 \times 10^{-3} M$ KCN; $4.0 \times 10^{-3} M$ acetanilide; $2.0 \times 10^{-3} M$ nicotinamide; rat liver microsomes (washed), 3 to 4 mg. protein/1 ml.; $37^\circ C$.; time of incubation, 60 min. Activities expressed in μ moles p -hydroxyacetanilide (determined by Folin-Ciocalteu reaction) formed per minute per milligram protein. Mean of 7 experiments.

imal and the incubation.* On the other hand, it must be mentioned that in the laboratories an activating effect of the ascorbic acid on steroid hydroxylation could not be seen, possibly because of different techniques.^{20,21,22} We too failed in demonstrating a positive effect of ascorbic acid on 11 β -hydroxylation, using an 11 β -hydroxylase preparation† instead of intact mitochondria.* The well-known effect of TPNH is inhibited by ascorbic acid (Hayano *et al.*;²³ Leonhäuser and Staudinger*). The more simple 21-hydroxylation of 17-hydroxyprogesterone to 11-desoxycortisol, which is known to proceed in adrenal microsomes with TPNH as a cofactor (Ryan *et al.*²⁴), is more suitable for studying the influence of ascorbic acid in steroid hydroxylation. DPNH can replace TPNH to some extent in this hydroxylation reaction (Leonhäuser and Staudinger*). Ac-

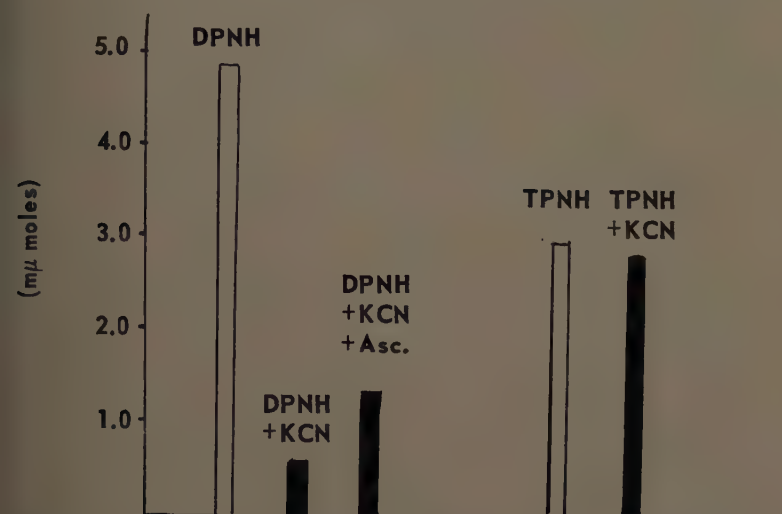


FIGURE 12. Influence of cyanide on the oxidation of TPNH and DPNH by rat liver microsomes (optical test): 0.06 *M* phosphate buffer; pH 7.4; 4.0×10^{-4} *M* TPNH; 4.0×10^{-4} *M* DPNH; 1.0×10^{-3} *M* KCN. Rat liver microsomes (washed) 0.35 mg. protein/1.25 (biuret); 37° C.; time 6 min. Activities expressed in mμ moles oxidized nucleotide per minute per milligram protein. Mean of 6 experiments.

ording to the experimental conditions, ascorbic acid has a more or less pronounced influence on the DPNH-dependent 21-hydroxylation. Investigations on these questions are continuing, so it seems preferable not to discuss the different results at this time by relatively theoretical reflections. Much experimental work has still to be done in this field to elucidate the physiological role of ascorbic acid in the adrenal cortex, about which there can be no doubt. The formerly expressed opinion^{3,17} that ascorbic acid has something to do with hydroxylation can also be demonstrated in another system. The enzymic hydroxylation of aromatic compounds (as for example, acetanilide) was described by Mitoma *et al.*²⁵ This system also proved to be suitable in

Leonhäuser, S., Hj. Staudinger, 1960, unpublished.

We are indebted to J. K. Grant (Edinburgh, Scotland) for the supply with 11 β -hydroxylase preparations.

our experiments. The average values of seven preliminary experiments are shown in FIGURE 11.

The hydroxylation activity of rat liver microsomes with TPNH as cofactor for hydroxylation is of the same extent, as already known.²⁵ The rats have been treated I.P. with benzpyrene 24 hours before.²⁶ DPNH too has a distinct activity (39 per cent of the TPNH-dependent hydroxylation) that is enhanced by ascorbic acid (50 per cent of the TPNH-dependent hydroxylation). Ascorbic acid alone has no effect on hydroxylation of acetanilide. The effect of DPNH and ascorbic acid can be demonstrated only when cyanide is present in a concentration of about 10^{-3} M. Without cyanide, the DPNH is rapidly oxidized by rat liver microsomes through a cyanide-sensitive pathway, involving probably cytochrome a_3 , which has been found in microsomes; TPNH oxidation by rat liver microsomes is not even inhibited by cyanide. This is also true, as already stated, for the ascorbic acid-dependent DPNH oxidation (FIGURE 12).

Apparently only cyanide-insensitive microsomal pathways of the hydrogen can serve for the hydroxylation reaction. As demonstrated, this is the case in the microsomal TPNH oxidation and, to a smaller extent, in a part of the microsomal DPNH oxidation and, last but not least, in the microsomal ascorbic acid-dependent DPNH oxidation. We are now investigating the assumption that cytochrome b_5 is also involved in this reaction. In this connection it must be emphasized that the demonstrated microsomal hydroxylation by DPNH and ascorbic acid proved to be really enzymatic and not, as one could perhaps assume, a variation of the well-known nonenzymatic hydroxylation system of Udenfriend *et al.*²⁸ and Brodie *et al.*²⁹ It must be stated, however, that the effect of ascorbic acid on hydroxylation of acetanilide is not very impressive. So it remains doubtful if this effect has anything to do with the hydroxylation of aromatic and other compounds *in vivo*. On the other hand one might remember that, in scurvy, hydroxylation of these compounds was decreased *in vivo*. (Axelrod *et al.*³⁰). Only further investigation can prove the validity of our opinion that the ascorbic acid-dependent electron transfer mechanism in microsomes is generally coupled with hydroxylation reactions.

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THE ROLE OF ASCORBIC ACID IN THE OXIDATION OF TRYPTOPHAN TO 5-HYDROXYTRYPTOPHAN

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It has been known for some time that the precursor of serotonin (5-HT) is tryptophan and that this metabolic conversion occurs in two steps. The first step is the hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP), while the second involves the decarboxylation of this hydroxyamino acid to 5-HT. The 5-HTP decarboxylase has been isolated from guinea pig kidney and partially purified by Clark *et al.*¹ Mitoma and his associates² have indicated the presence of the hydroxylating system in whole-cell studies of *Chromobacterium violaceum*, and Schindler³ has demonstrated the presence of the enzyme system in neoplastic murine mast cells. However, attempts to isolate and characterize the hydroxylating system in nonneoplastic mammalian tissue have thus far been unsuccessful.⁴

This report is concerned with the isolation from normal tissues of both the rat and the guinea pig and the partial characterization of the enzyme system that catalyzes the hydroxylation of tryptophan to 5-hydroxytryptophan. The enzyme, which will be provisionally referred to as tryptophan-5-hydroxylase, has been shown to require for its activity both cupric ions and ascorbic acid.

The hydroxylation reaction was assayed by adding the 5-HTP decarboxylase in excess in order that all the 5-HTP formed would be converted to 5-HT. D,L-Tryptophan-3-C¹⁴ was used as the substrate. With the use of a weak cation-exchange resin,⁵ tryptophan was separated from 5-HT, and the latter was then assayed by radioactivity measurements. That this radioactivity was attributable to C¹⁴-5-HT was established by paper chromatography.

The composition of the incubation medium is shown in TABLE 1. With regard to the enzyme, the hydroxylating activity is found in the intestinal mucosal cells and, to a small extent, in the kidney, as shown in TABLE 2. Liver, brain, pineal gland, large intestine, and spleen extracts did not contain any measurable quantity of tryptophan-5-hydroxylase. The enzyme system is located in the particulate fraction of the intestinal mucosal cell, but no discrete localization of activity within the particulate elements was observed; thus approximately equal activity was observed when nuclei, mitochondria, and microsomes were incubated with substrate and appropriate cofactors. On the other hand, not only was the soluble fraction of the cell devoid of tryptophan-5-hydroxylase but it appeared to contain an inhibitor of this activity. Thus using an unfractionated homogenate as the enzyme source, only about 25 per cent of the activity was obtained as compared with that seen when the particulate fraction of the mucosal cells served as the source of the enzyme. Similarly the addition of a dialyzed aliquot of the soluble fraction of the cell to a particulate preparation markedly inhibited the hydroxylation of tryptophan to 5-HTP. The nature of this inhibitor is still unknown; its presence may partially explain the difficulties encountered by previous investigators in finding this enzyme activity.

In TABLE 3 are shown the dependencies of the hydroxylation reaction on Cu^{++} and ascorbic acid. Although an extensive search was conducted for other factors, no other stimulatory substances were uncovered. The requirement for Cu^{++} cannot be replaced by Cu^+ , Fe^{+++} , Mo^{VI} , or Mn^{++} . With respect to the requirement for ascorbic acid, D-ascorbic acid is as active as the L-form, while

TABLE 1
INCUBATION MEDIUM

Phosphate buffer, pH 7.4.....	150 μ moles
L-Tryptophan.....	100 μ g.
C^{14} -D,L-Tryptophan.....	6×10^6 cpm
5-HT.....	200 μ g.
Pyridoxal phosphate.....	0.2 μ mole
Ascorbic acid.....	2 μ moles
Cupric chloride.....	4 μ moles
Kidney supernate*.....	0.1 ml.
Particulate preparation†.....	2 ml.
Water to give a final volume of 3 ml.	

Incubation was carried out at 37° C. for 60 min. in a Dubnoff shaking incubator.

* Guinea pig or rat kidney was homogenized with 3 volumes of water, centrifuged at 5,000 g, and the supernatant fraction was used as a source of 5-HTP decarboxylase.

† Guinea pig or rat intestinal mucosal cells were homogenized with 4 volumes of 0.01 M phosphate buffer, pH 7.4, and centrifuged at 78,000 g. The supernatant fraction was discarded, and the particulate fraction was resuspended with water in an amount sufficient to give the volume of the original homogenate.

TABLE 2
TISSUE LOCALIZATION OF TRYPTOPHAN-5-HYDROXYLASE

Tissue	Enzyme activity*
Small intestine	++++
Kidney	+
Liver	—
Brain	—
Large intestine	—
Spleen	—
Pineal	—

* Incubation conditions described in TABLE 1. The particulate fraction of the tissue homogenates was used as a source of tryptophan-5-hydroxylase. Symbols: + = activity; — = no activity.

TABLE 3
DEPENDENCIES OF THE HYDROXYLATION SYSTEM*

Additions	cpm
Complete	7735
- Cu^{++}	645
-Ascorbate	125
-Enzyme	775

Incubation conditions as described in TABLE 1.

the substitution of dehydroascorbic acid or isoascorbic acid for ascorbic acid generally resulted in an even higher rate of tryptophan hydroxylation. Thus although specificity within the ascorbic acid group is lacking, attempts to replace ascorbic acid with cytochrome c, flavin adenine nucleotide (FAD), flavin mononucleotide (FMN), or the pyridine nucleotides, or with artificial electron acceptors such as methylene blue, dichlorophenolindophenol, or the tetrazolium dyes were unsuccessful.

It is also to be noted in TABLE 3 that in the absence of the hydroxylating system, but in the presence of 5-HTP decarboxylase, some 5-HT is produced. That the radioactivity measured in this experiment was, in fact, 5-HT was shown by paper chromatography. This nonenzymic hydroxylation occurred to a variable degree; the amount ranged from less than 10 per cent of the hydroxylation in the presence of tryptophan-5-hydroxylase in some experiments to 50 per cent of the enzyme-catalyzed reaction in others. Hydroxylation of tryptophan in the absence of the enzyme system was very sensitive to the pH of the incubation medium. Thus at pH 7.0, enzyme activity was reduced by 10 per cent, while nonenzymic activity dropped 50 per cent; at pH 6.5, a 60 per cent decline in tryptophan-5-hydroxylase activity was noted, whereas the nonenzymic reaction was completely obliterated. It was also observed that the hydroxylation in the absence of enzyme occurred under anaerobic conditions.

Under the usual incubation procedure (using 100 $\mu g.$ of tryptophan) and in the presence of an excess of 5-HTP decarboxylase, the conversion of tryptophan to 5-HT proceeded at a linear rate for 60 min., the usual incubation time, and resulted in the formation of 2 to 7 $\mu g.$ of 5-HT. Thus the hydroxylation step is most certainly the rate-limiting reaction in the formation of 5-HT. It may well be that, because of the presence of an inhibitory factor in the soluble portion of the cell, the rate of the hydroxylation reaction *in vivo* may be even slower than that indicated above.

One of the most interesting findings in this study was that the hydroxylation reaction proceeds equally well under either anaerobic or aerobic conditions. To our knowledge this is the first demonstration of an aromatic hydroxylation reaction in mammalian tissues that takes place under conditions of anaerobiosis. This finding, coupled with the observation that catalase does not inhibit the reaction, indicates that hydrogen peroxide is not involved in the reaction. With respect to the mechanism of this hydroxylation, it is possible that a hydride ion is removed from the 5-position on the tryptophan ring, and that this is replaced by OH^- from the water; the resulting two protons and two electrons would then reduce the true electron acceptor in this reaction, dehydroascorbic acid, to ascorbic acid. The cycle would be completed by the oxidation by copper of ascorbic acid to dehydroascorbic acid. No definitive answer concerning the mechanism of this hydroxylation can be given until a greater degree of purification of the enzyme system has been attained.

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ON THE INACTIVATION OF ASCORBIC ACID OXIDASE*

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The enzyme ascorbic acid oxidase (AAO) is a blue, copper-containing protein that catalyzes the aerobic oxidation of L-ascorbic acid to dehydroascorbic acid. The reaction is readily followed manometrically, and when pure (catalase free) AAO is employed the enzymatic oxidation is easily differentiated from the cupric-ion-catalyzed reaction by the oxygen stoichiometry. The complete oxidation of L-ascorbic acid as catalyzed by the enzyme requires the consumption of 1 gram atom of oxygen, which is reduced to water. The oxidation when catalyzed by free cupric ion, however, requires 1 mole of oxygen (2 gram atoms) per mole of ascorbic acid, and hydrogen peroxide is readily detected as a terminal product (see FIGURE 1).

The blue color of the purified enzyme is one of its most characteristic properties. The intensity of the color per unit weight of copper is very much greater than that of solutions of copper sulfate or of the copper-ammonia complex.¹ The copper in the enzyme protein-copper complex is very tightly bound.² It is not removed by dialysis at physiological pH, nor by treatment with ion exchange resins.³ Furthermore, the copper of the resting (nonfunctioning) enzyme does not exchange with radioactive (Cu^{64}) cupric ions when highly purified enzyme is employed.^{4,5} The activity of the enzyme is critically dependent on its copper content, for when the copper is removed by dialysis against cyanide the activity is simultaneously lost (FIGURE 2).

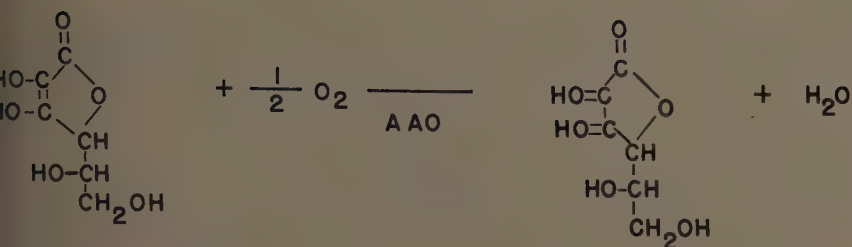
The properties of the purified enzyme are those of a globular protein having a molecular weight of 150,000 and containing approximately 0.26 per cent copper, corresponding to 6 copper atoms per molecule. Additional information about the purified enzyme is summarized in TABLE 1.

One of the most characteristic properties of this copper oxidase is the marked inactivation of the enzyme that occurs during the catalytic oxidation of the substrate. The nature of the phenomenon is indicated in FIGURE 3. The oxygen-uptake curves shown in FIGURE 3 are typical of those obtained when a purified sample of the enzyme is employed. When a relatively large amount of the enzyme is used, a complete oxidation of the substrate occurs. However, when a smaller amount of the enzyme is employed, the rate of the oxidation reaction rapidly and continuously decreases until the enzyme is completely inactivated. The "reaction inactivation" of the enzyme is indicated by an "inactivation total," that is, an incomplete oxidation of the ascorbic acid as measured by oxygen uptake. The reaction inactivation of the enzyme is irreversible, and further oxidation of the residual ascorbic acid does not occur unless more enzyme is added.

A number of years ago it was found in our laboratory that inert protein, such as gelatin, protected the enzyme against inactivation; and certain agents, such as native catalase and peroxidase, native or denatured methemoglobin, and

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ENZYME-CATALYZED OXIDATION OF L-ASCORBIC ACID



CUPRIC ION-CATALYZED OXIDATION OF L-ASCORBIC ACID

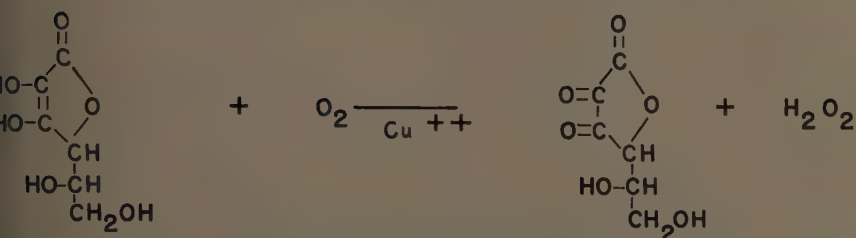


FIGURE 1. The aerobic oxidation of ascorbic acid as catalyzed by AAO and by free cupric ion.

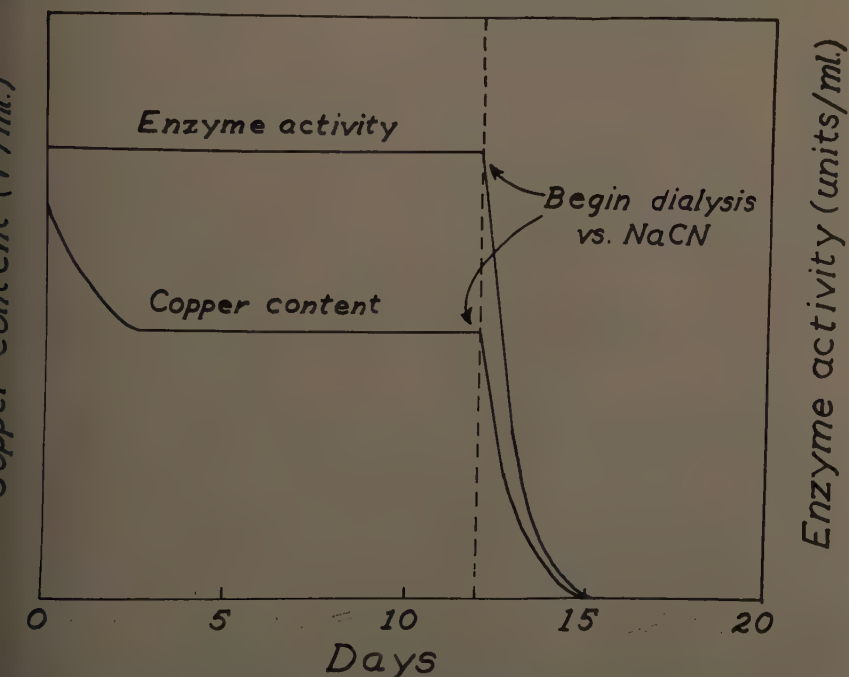


FIGURE 2. Showing the stability of the enzyme's copper content and activity to dialysis at physiological pH. Horizontal scale gives the days of dialysis. Note that both the activity and copper content are rapidly lost when cyanide is added to the system.

hematin, were strikingly effective in protecting the enzyme against reaction inactivation.⁷ In other words, these agents were found to increase markedly the inactivation total per unit of enzyme. These studies in 1944 also indicated that environmental factors such as the rate of shaking of the manometers, the

TABLE 1
ASCORBIC ACID OXIDASE⁶

Source: summer crookneck squash (<i>Curcubita pepo condensa</i>)
Preparation: differential ultracentrifugation and fractional precipitation by dialysis
Specific activity: 2000 U./mg.
Color: blue-green (dependent on O ₂ and substrate)
Homogeneity: 100 per cent (ultracentrifuge and electrophoresis)
Molecular weight: 150,000
Copper: 0.26 per cent, corresponding to 6 copper atoms per mole
Activity per copper atom: 740 U.
Nitrogen: 16.8 per cent; no P or Ca

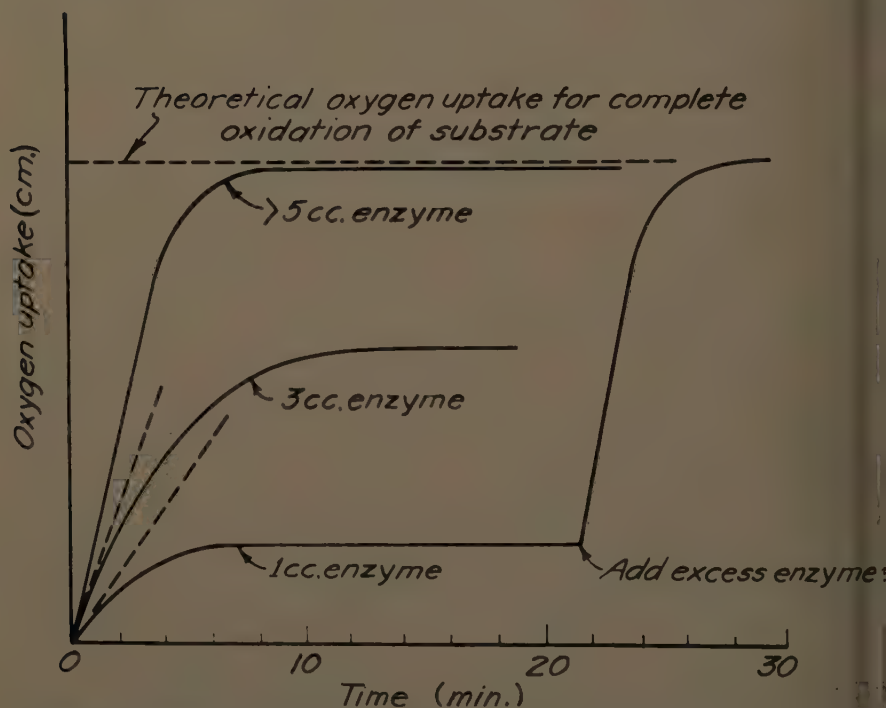


FIGURE 3. Showing how reaction inactivation becomes evident when relatively smaller amounts of the enzyme are used.

pH of the system, the substrate concentration, and the enzyme concentration were minor factors in the reaction inactivation. Because earlier experiments in our laboratory⁸ had shown that hydrogen peroxide was not a terminal product of the ascorbic acid-AAO reaction, and the main product dehydroascorbic acid

was not harmful to the enzyme, it was suggested⁷ that the reaction inactivation might be due to some factor inherent in the ascorbic acid-AAO-oxygen system, possibly a highly reactive "redox form" of oxygen other than hydrogen peroxide or H_2O_2 .

The experimental work in 1944 also demonstrated that the inactivation total, for any given amount of enzyme, decreased as the substrate concentration was increased, but this inhibitory effect of the substrate was not further explored at that time.

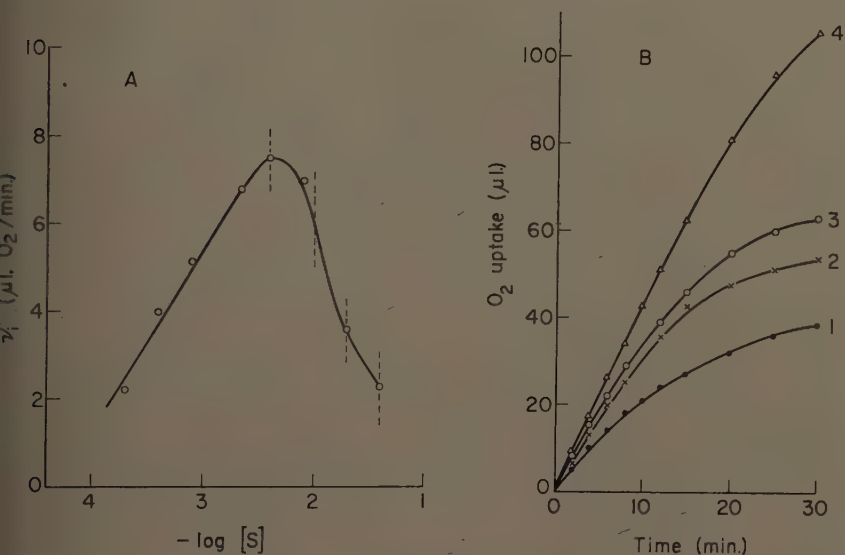


FIGURE 4A. Showing how the apparent initial reaction velocity varies with the substrate concentration, expressed as $-\log (S)$.

B. Showing how the apparent initial reaction velocity and the reaction course vary with increasing substrate concentration: curve 1: $4 \times 10^{-2} M$ ascorbic acid; curve 2: $2 \times 10^{-2} M$ ascorbic acid; curve 3: $1 \times 10^{-2} M$ ascorbic acid; curve 4: $4 \times 10^{-3} M$ ascorbic acid.

Oxygen uptake was measured manometrically at 25°C. , $\text{pH } 5.8$. Each vessel contained O ($0.4 \mu\text{g.}$), $4 \times 10^{-2} M$ citrate-phosphate buffer, ascorbic acid, and distilled water to make a final reaction volume 2.5 ml.

The substrate concentrations involved in FIGURE 4B are shown as dotted lines in FIGURE

During the past year we have carefully re-examined the possibility that substrate inhibition might be an important factor in the reaction inactivation of ascorbic acid oxidase. Our work has revealed that the reaction inactivation can be differentiated from substrate inhibition. The major factor responsible for the loss in activity of the enzyme during its catalytic function appears to be the alteration of the enzyme by very small amounts of hydrogen peroxide produced by a secondary reaction during the enzymatic oxidation of the ascorbic acid. Some of the experimental evidence in support of this conclusion may now be reviewed.

The curves in FIGURE 4 show how the activity of the enzymes decreases (FIGURE 4A) and the inactivation of the enzyme increases (FIGURE 4B) as the sub-

strate concentration is increased beyond the optimum. It is apparent from FIGURE 4 that substrate inhibition significantly increases the reaction inactivation; this is shown even more strikingly by the curves of FIGURE 5. In the experiment corresponding to curve 1, the total substrate was added at zero time; it was an amount of substrate that resulted in a substrate concentration slightly higher than the optimum. When the experiment was repeated under the same conditions of enzyme concentration (except that the same total amount of substrate was added stepwise, as indicated, in six equal increments so that the substrate concentration was always below the optimum), curve 2 was the result. The enzyme accomplished much more oxidation before it became completely inactivated, which was after the sixth substrate addition.

Although it is apparent from FIGURES 4 and 5 that the effect of substrate inhibition on the reaction inactivation is pronounced, it is also clear from curve 2 of FIGURE 5 that the inactivation is readily apparent even in the absence of substrate inhibition. The loss in enzyme activity during the reaction must therefore be due to some other factor or factors.

The experimental results indicated by the curves of FIGURE 6 establish that the reaction inactivation cannot be ascribed to the products of the enzymatic reaction or to any intermediates or secondary substances with concentration at any given time dependent on the initial enzymatic reaction rate. The stepwise addition of the substrate in the experiment corresponding to curve 2 resulted in a lower rate of oxidation, as expected, but the rate and extent of enzyme inactivation was not correspondingly decreased; rather it was increased. Consequently it may be concluded that the inactivation cannot be due to any factor with a concentration dependent on the rate of the enzymatic oxidation.

Much new information about the nature of the inactivation phenomenon and its cause was revealed when the enzyme was added incrementally to a given amount of substrate (FIGURE 7).

In experiments 1 and 2 of FIGURE 7 the same quantities of ascorbic acid were used. The total amounts of enzyme used were also the same; in experiment 1 however, the total enzyme was added initially, whereas in experiment 2 the enzyme was added at the periods indicated in increments of one fifth the amount used in experiment 1. Only in the experiment involving the increment additions (curve 2) was enzyme inactivation apparent. Furthermore the apparent enzyme inactivation was about the same for the third, fourth, and fifth enzyme increments. In the case of the first enzyme increment the rate of the oxidation was significantly lower than with the later increments. This was undoubtedly the result of substrate inhibition, as discussed earlier.

In the experiment corresponding to curve 3, the amount of substrate was one half that involved in experiments 1 and 2. The same quantity of enzyme however, was used as in each increment addition in experiment 2. It is to be noted that the time required for enzyme inactivation was very similar to that required for each enzyme increment in experiment 2. However, it is evident from curve 3 that the enzyme accomplished much more oxidation before it became inactivated. This point is particularly apparent if curve 3 is compared with the O_2 -uptake curve resulting after the fifth enzyme increment addition in experiment 2, where at the time of addition (75 min.) the amount of residual

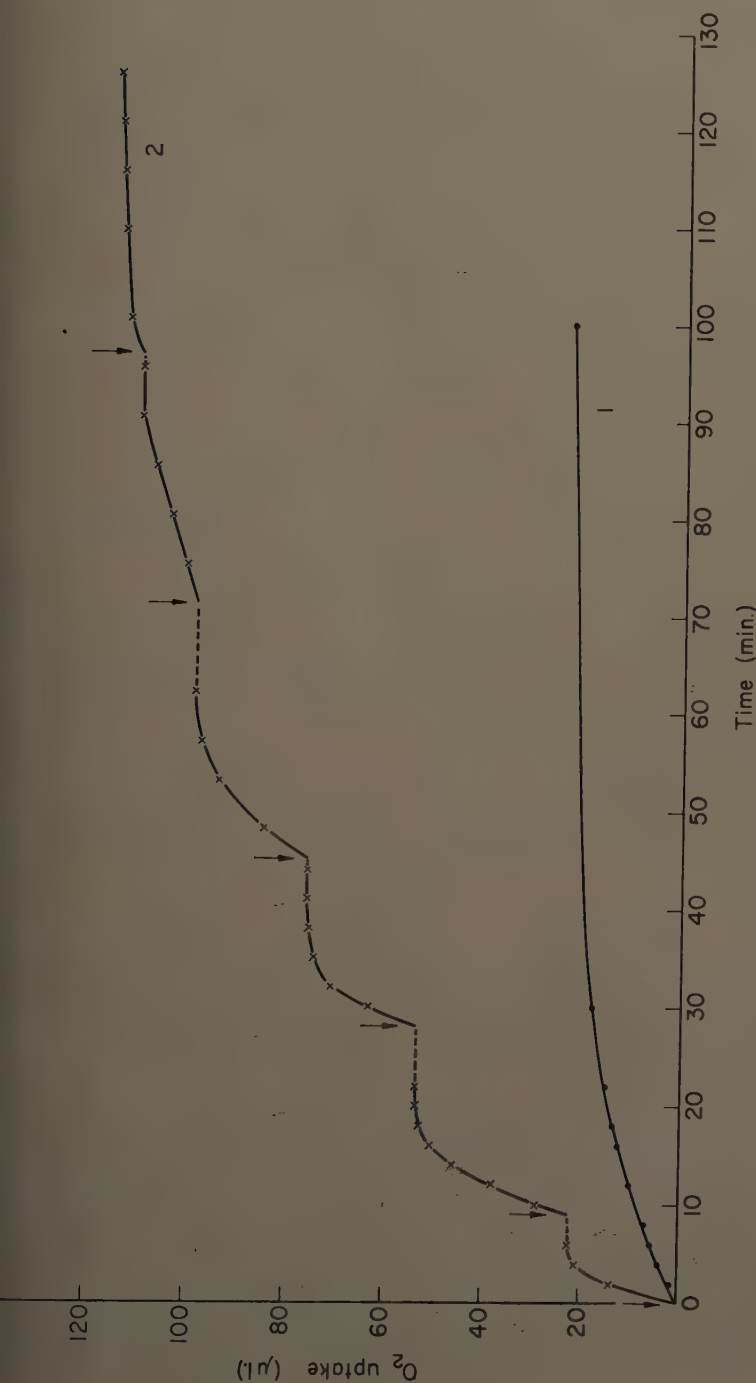


FIGURE 5. Showing how the stepwise addition of concentrated substrate increases the apparent activity of the enzyme. Oxygen uptake was measured manometrically at 25° C., pH 5.8. Each vessel contained AAO (0.4 μg.) 4×10^{-2} M citrate-phosphate buffer, 6×10^{-3} M ascorbic acid and distilled water to make the final reaction volume 2.5 ml. In the experiment corresponding to curve 1, the total substrate was added at zero time, while in experiment 2, the substrate was added in increments (total/6) as indicated.

unoxidized substrate was approximately one half the initial substrate concentration (comparable to that used in experiment 3).

The lower rate and extent of oxidation resulting after the fifth enzyme increment in experiment 2 (as compared with experiment 3) would suggest the accumulation of an effective inactivating agent (such as H_2O_2) during the previous 75 min. of the stepwise oxidation procedure used in experiment 2.

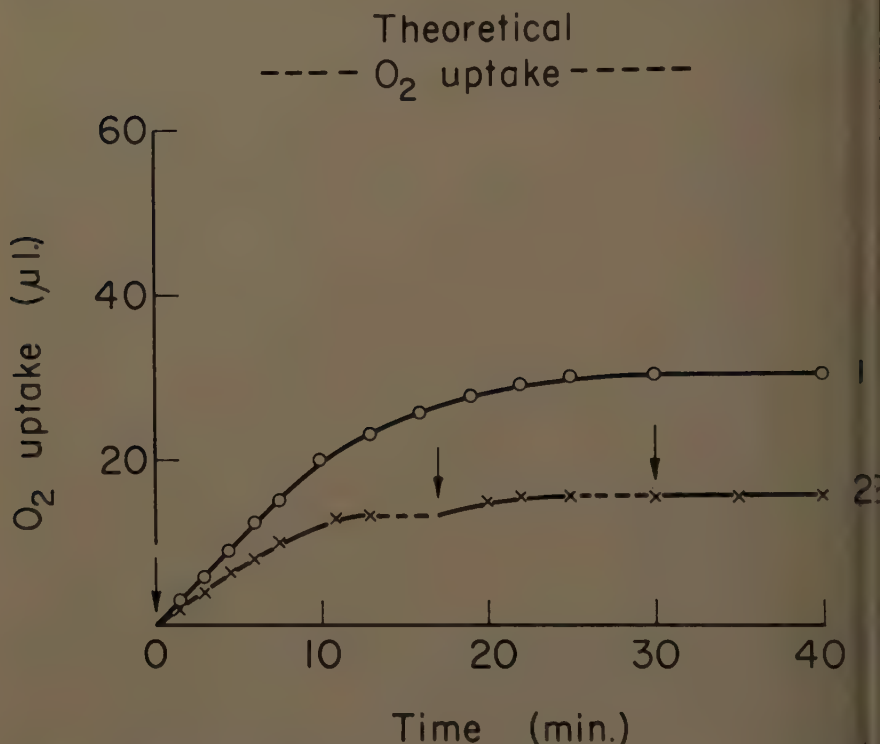


FIGURE 6. Showing how the stepwise addition of diluted substrate decreases the apparent activity of the enzyme.

Oxygen uptake was measured manometrically at 25° C., pH 5.8. Each vessel contained AAO (0.4 μ g.) 4×10^{-2} M citrate-phosphate buffer, 2.4×10^{-3} M ascorbic acid, and distilled water to make the final volume 2.5 ml. In the experiment corresponding to curve 1, the total substrate was added at zero time, while in the experiment 2, the substrate was added in increments (total/3) as indicated.

In the case of experiment 2, a sixth increment of the enzyme was added at the 95-min. period; it was found that the extent of oxidation accomplished by the enzyme and the time required for its inactivation was less than the previous increments. Such results would be anticipated for a system gradually accumulating an enzyme-inactivating agent such as H_2O_2 .

To check the possibility that simple autoxidation of the substrate might be producing sufficient H_2O_2 to account for the enzyme inactivation, the effect of exposing the substrate to the reaction conditions in the absence of enzyme for

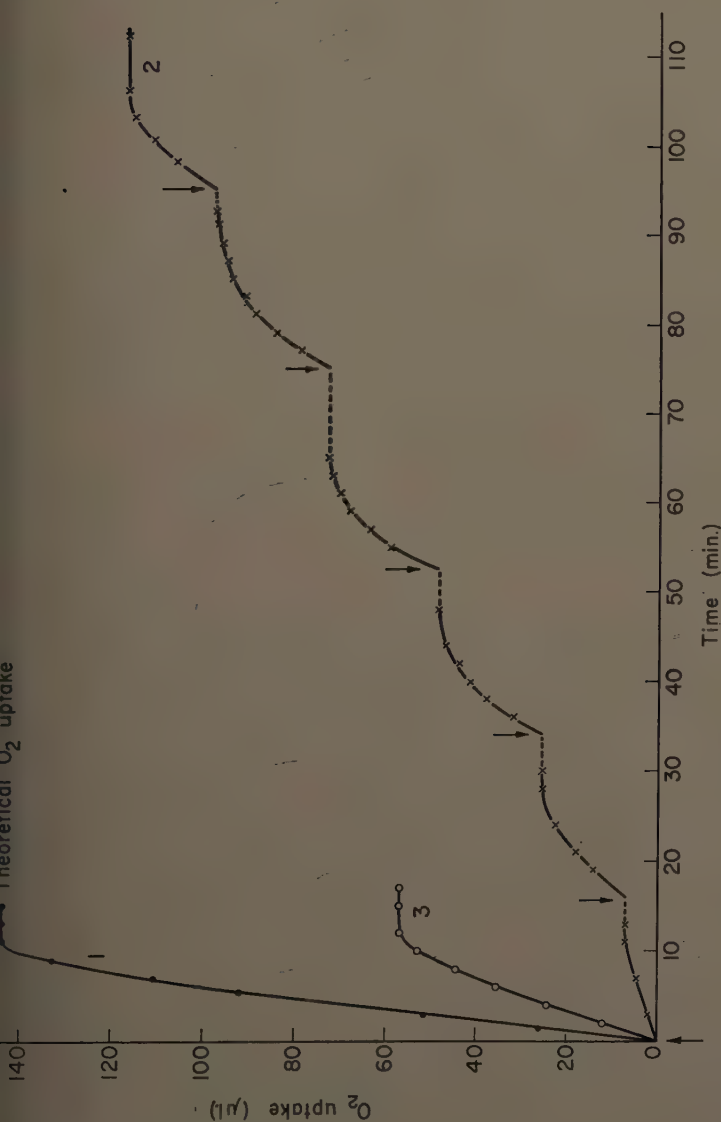


FIGURE 7. Showing how the enzyme inactivation depends on the ratio of enzyme to substrate and the time of the reaction.

Oxygen uptake was measured manometrically at 25°C , pH 5.8. In experiments 1 and 2, each vessel contained AAO ($2\text{ }\mu\text{g}$), $4 \times 10^{-2}\text{ M}$ citrate-phosphate buffer, $4.8 \times 10^{-3}\text{ M}$ ascorbic acid, and distilled water to make the final reaction volume 2.5 ml. In experiment 1, the total enzyme was added at zero time; in experiment 2, each increment of AAO (total/5) was added as indicated. In experiment 3, the reaction vessel contained AAO ($0.4\text{ }\mu\text{g}$), $4 \times 10^{-2}\text{ M}$ citrate-phosphate buffer, $2.4 \times 10^{-3}\text{ M}$ ascorbic acid, and distilled water to make the final volume 2.5 ml. The enzyme (total/5) was added at zero time.

varying periods of time before initiating the enzymatic oxidation (by adding the enzyme) was investigated. It was found through such pre-incubation experiments that the initial reaction velocities, inactivation totals, and inactivation times were not significantly different. In other words, it could be concluded that simple autoxidation of the substrate in the absence of enzyme could not account for the reaction inactivation phenomenon.

In view of the evidence that a gradually accumulating by-product in the re-

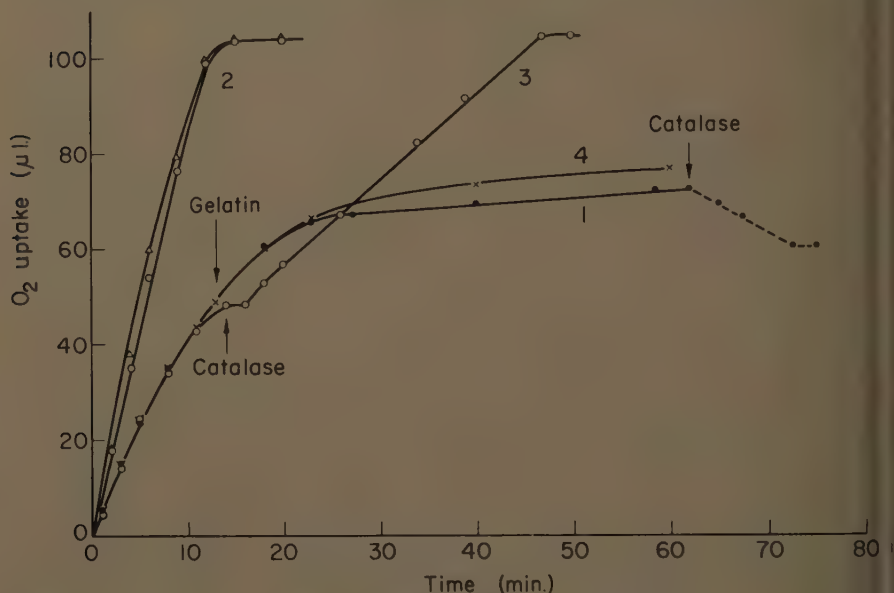


FIGURE 8. Showing the effects of adding catalase and gelatin at various times on the reaction course.

Oxygen values were measured manometrically at 25° C., pH 5.8. In addition to the components shown below, each vessel contained AAO (0.4 μg.), 4×10^{-2} M citrate-phosphate buffer, 4×10^{-3} M ascorbic acid and copper-free water to make the final reaction volume 2.5 ml.

Experiment 1: no catalase or gelatin initially. 18 μg. catalase was added later as indicated; experiment 2: 18 μg. catalase or 1 mg. gelatin was added to the buffered enzyme system before initiating the reaction by addition of substrate; experiment 3: 18 μg. catalase was added during the reaction as indicated. Experiment 4: 1 mg. gelatin was added during the reaction as indicated.

action, such as H_2O_2 , might be responsible for the enzyme inactivation, it seemed advisable to reinvestigate carefully the effects of adding catalase and gelatin (separately) before, during, and after the enzymatic reaction. The results are shown in FIGURE 8. Curve 1 (the control) shows that under the conditions of the experiment, the enzyme in the absence of protective agents was inactivated when only about 70 per cent of the ascorbic acid had been oxidized. Complete oxidation of the ascorbic acid was rapidly obtained when a very small amount of catalase, or a considerably larger amount of gelatin, was added initially to the otherwise identical enzyme-ascorbic acid system (curve 2). When the experiment was repeated (except that the same amount of catalase

was added after the oxidation had progressed for about 12 min.), a slight lag in oxygen uptake occurred (curve 3); then the rate became essentially linear (no further inactivation) until the ascorbic acid was completely oxidized. However, when gelatin was added during the course of the reaction (curve 4) there was only a slight protective effect. It should be noted that in the control experiment (curve 1) catalase was added at the 62-min. period after the enzyme had been completely inactivated and a small evolution of gas had resulted (presumably due to hydrogen peroxide decomposition). The addition of gelatin at this point was without effect. Furthermore, the addition of catalase after the rapid and complete oxidation of ascorbic acid by an excessive amount of ascorbic acid oxidase never resulted in any evidence of hydrogen peroxide accumulation.

The experimental results depicted in FIGURE 8 establish that the effectiveness of catalase as a protective agent against the reaction inactivation of AAO is very much greater than that of gelatin. The nature of the protective effect, as well as the gas evolution observed when the catalase was added after the reaction had proceeded for a period, are evidence that the catalase effect is the result of its destruction of small amounts of hydrogen peroxide. The hydrogen peroxide production cannot be a result of the main enzymatic reaction, for if such were the case the reaction inactivation would be expected to vary directly with the rate of the oxidation (FIGURE 6). It therefore appears that the inactivation is due to very small amounts of hydrogen peroxide resulting from a lower and secondary reaction. The gelatin protective effect can also be rationalized in terms of hydrogen peroxide production. It is known that the effect is observed with other proteins; it seems likely, therefore, that the gelatin simply reacts chemically with the hydrogen peroxide in a rather slow and ineffective manner. When the gelatin is added initially it can cope with the slow production of hydrogen peroxide and thereby protect the enzyme if a relatively large amount (in comparison to the enzyme protein) is present. However, when gelatin is added to the system after a certain amount of excess hydrogen peroxide has accumulated, the gelatin cannot neutralize the hydrogen peroxide rapidly enough to prevent effectively the continuing inactivation of the enzyme (curve 4, FIGURE 8).

It has been found that only very small amounts of hydrogen peroxide are necessary to account for the observed enzyme inactivation. Furthermore, additional experimental results have been obtained* that support the conclusion that the hydrogen peroxide production is not a result of the main enzymatic reaction but involves part of the protein-bound copper in a secondary way. In other words, it is suggested that all of the copper in the enzyme may not be simultaneously involved in the enzymatic activity.

Summary

During the aerobic oxidation of ascorbic acid catalyzed by the copper protein, ascorbic acid oxidase, the enzyme is rapidly and extensively inactivated. The phenomenon has been reinvestigated, and new evidence concerning the

* K. Tokuyama and C. R. Dawson. 1961. "On the Mechanism of the Reaction Inactivation of Ascorbic Acid Oxidase." To be published.

cause of the reaction inactivation has been obtained. It has been found that the enzyme inactivation is dependent on the time during which a secondary product of the reaction may accumulate. The main products of the enzymatic oxidation, that is, dehydroascorbic acid and water, appear not to be involved in the inactivation phenomenon. However, a secondary product, hydrogen peroxide, develops in small amounts during the reaction as the result of non-enzymatic copper protein catalysis. In other words, it appears that some of the copper sites on the enzyme protein function nonenzymatically in the sense that they produce the secondary product, hydrogen peroxide, at a low rate. Hydrogen peroxide is a very effective inactivating agent against ascorbic acid oxidase, and only very small amounts are required to account for the experimentally observable enzyme inactivation.

Acknowledgment

We are indebted to Stanley Lewis for technical assistance.

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ROLE OF ASCORBIC ACID IN THE INCORPORATION OF PLASMA IRON INTO FERRITIN*

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During the course of studies concerned with the biochemical mechanism responsible for the transfer of plasma-bound iron to the liver and its incorporation into ferritin in this tissue, we¹ obtained evidence for the participation of two compounds in this reaction, adenosine triphosphate (ATP) and ascorbic acid. Since ascorbic acid is rather specific for this reaction and cannot be replaced by the usual biochemical reducing agents, we¹ believe that we have found a unique biochemical function for ascorbic acid. I shall present in this paper the experimental evidence for the participation of these compounds in the iron incorporation reaction as well as data that may be helpful in formulating a mechanism for the reaction.

Plasma-bound iron exists in the form of an iron-protein complex called transferrin, capable of combining with a maximum of 2 iron atoms per molecule, but which in the normal plasma is one third saturated with respect to iron. The iron atoms are tightly bound to the protein and are released after reduction to the ferrous state at acid pH . At neutral pH , reducing agents such as ascorbic acid are without activity, especially at the low concentrations present in plasma. Iron in ferritin is present for the most part as colloidal micelles of ferric hydroxide-ferric phosphate, constituting about 17 to 25 per cent by dry weight of the protein. While most of the iron appears to reside inside the molecule, surrounded by a protein envelope, some iron at or near the surface of the protein, is available for reaction with mild reducing agents at neutral pH , and is probably in equilibrium with the bulk of ferritin iron inside the protein molecule. The biochemical mechanism responsible for the release of ferritin-bound iron of the liver involves a reaction of its surface iron with the reduced form of the enzyme, xanthine oxidase, leading to its release and subsequent binding by plasma transferrin.^{2,3}

Experiments with surviving liver slices (TABLE 1) indicate that energy derived from oxidative metabolism is required for the incorporation of serum-bound iron into ferritin of the hepatic cell. I should like to emphasize the fact that for this type of experiment it is absolutely necessary to use the physiological form of plasma iron as opposed to inorganic iron compounds. Results obtained with inorganic iron added to buffered systems at pH 7.4 are quite different from those obtained with transferrin-bound iron⁴ and have little meaning with respect to an understanding of the biochemical processes that occur. Inorganic iron, added to a neutral solution containing cellular material, combines nonspecifically with all proteins, with acids, phenols, and with inorganic phosphate. In our experiments, the incorporation reaction requires oxygen; it is markedly inhibited by anaerobic conditions, although the quantity of iron bound to the liver cell is the same as in the presence of oxygen. This suggests

* The investigation reported in this paper was supported in part by Grant A-1655 from the National Institutes of Health, Public Health Service, Bethesda, Md.

that whereas the binding site for plasma iron is not associated with energy-yielding reactions, the incorporation phenomenon requires energy.

Further confirmation of the need for oxidative metabolism during the incorporation reaction is demonstrated in TABLE 2. Substrates for a variety of oxidative enzymes stimulate the incorporation of serum Fe^{59} into ferritin of

TABLE 1*
INCORPORATION OF PLASMA Fe^{59} BY LIVER SLICES†

Time (min.)	Oxygen			Nitrogen		
	Radioactivity of		Ferritin iron ($\mu\text{g.}$)	Radioactivity of		Ferritin iron ($\mu\text{g.}$)
	Liver	Ferritin		Liver	Ferritin	
	<i>cpm/gm. liver</i>			<i>cpm/gm. liver</i>		
30	602	211	88	705	32	72
60	766	288	80	962	34	53
90	862	473	82	1131	29	42
120	1050	550	83	1231	38	31

* Reproduced by permission from *The Journal of Biological Chemistry*.¹

† Each incubation flask contained 2.0 gm. of pooled rat liver slices, 10 ml. of Krebs-Ringer-bicarbonate and 0.5 ml. of diluted rat plasma tagged with 100,000 cpm of Fe^{59} . Incubation was carried out at 37° C. in an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide, or 95 per cent nitrogen and 5 per cent carbon dioxide. The slices were washed 3 times with 50-ml. portions of 0.9 per cent sodium chloride solution before removal of aliquots of 0.5 gm. for counting and 1.0 gm. for ferritin isolation.

TABLE 2*
EFFECT OF SUBSTRATES AND INHIBITORS ON PLASMA Fe^{59} INCORPORATION
INTO LIVER SLICES†

Substrate	Radioactivity in		Inhibitor	Radioactivity in	
	Liver	Ferritin		Liver	Ferritin
Control	1.0	1.0	Control	1.0	1.0
Citrate	2.1	4.6	Iodoacetamide	1.2	0.1
Oxalacetate	1.8	2.7	N-Ethyl maleimide	1.0	0.1
Malate	1.5	2.7	Cyanide	1.1	0.2
Fumarate	1.5	2.4	Arsenite	1.0	0.2
α -Ketoglutarate	1.1	1.8	Dinitroresol	0.9	0.3
Succinate	1.0	1.8	Dinitrophenol	0.8	0.5
Pyruvate	1.0	1.8	Azide	1.2	0.5
Lactate	1.0	1.7			
Aspartate	1.0	1.6			
Glutamate	1.0	1.2			
Glucose	1.0	1.0			
β -Hydroxybutyrate	1.1	0.9			

* Reproduced by permission from *The Journal of Biological Chemistry*.¹

† Conditions the same as in TABLE 1, using oxygen-carbon dioxide. Control flasks contained no added substrate, and the Fe^{59} content of the total liver as well as the specific activity of the ferritin obtained from this control liver are adjusted to values of 1.0. Substrates were present at a final concentration of 0.01 M, whereas inhibitors were present at a final concentration of 5×10^{-3} M.

erobic liver slices, whereas marked inhibition of this reaction occurs in the presence of inhibitors of respiratory enzymes or of oxidative phosphorylation.

In order to identify those steps in oxidative metabolism that are most clearly associated with the iron incorporation reaction, we next studied the behavior of broken cell suspensions, homogenates. The results in TABLE 3 show that nicotinamide, which prevents the breakdown of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) by enzymes released by homogenization, preserves the activity of the suspension. Addition of DPN or TPN to such a system further enhances its activity, confirming the need for oxidative enzyme activity. Fluoride, which inhibits the activity of ATPase, is also stimulating, and addition of extra ATP itself causes a marked enhancement of the over-all activity, suggesting that ATP is directly involved in the iron incorporation reaction.

TABLE 3*

INCORPORATION OF SERUM Fe^{59} INTO FERRITIN OF LIVER HOMOGENATES†

Additions	Relative specific activity of ferritin Fe
ATP alone	1.0
Nicotinamide (0.1 M)	1.7
Nicotinamide (0.1 M) + DPN (3.4 μ moles)	2.0
Nicotinamide (0.1 M) + TPN (2.6 μ moles)	4.0
Nicotinamide (0.1 M) + F^- (0.1 M)	9.2
Nicotinamide (0.1 M) + F^- (0.1 M) + ATP (5 μ moles)	10.1
Nicotinamide (0.1 M) + F^- (0.1 M) + ATP (10 μ moles)	12.2
Nicotinamide (0.1 M) + F^- (0.1 M) + ATP (20 μ moles)	14.9

* Reproduced by permission from *The Journal of Biological Chemistry*.¹

† Each flask contained the equivalent of 1.0 gm. wet weight of rat liver in the form of a homogenate prepared with 0.15 M KCl-0.0006 M KHCO_3 and serum-bound Fe^{59} (50,000 c.p.m.). Flasks were incubated at 37° C. for 30 min. in an atmosphere of 100 per cent oxygen. Specific activities of ferritin iron of homogenates in the experimental flasks were compared with that isolated from the control flask, which was adjusted to 1.0.

ATP alone is ineffective when added to a purified system containing serum-bound Fe^{59} and ferritin. There is required, in addition, an extract of liver tissue that contains a soluble compound. As shown in TABLE 4, this compound is only slightly heat-labile but is completely dialyzable. Its identity with ascorbic acid was proved by use of ascorbic acid oxidase, kindly given to us by Dawson of Columbia University, New York, N.Y. We now possessed a system that was capable of incorporating serum-bound Fe^{59} into ferritin protein *in vitro*. TABLE 5 demonstrates the activity of such a purified system and shows further that maximum activity occurs at a molar ratio of ATP:ascorbic acid of 2:1. The reaction is linear with time and is not further activated by addition of liver extract.

For a study of the mechanism of the iron incorporation reaction, we may assume that the initial step involves the reduction of ferric iron, attached to transferrin, to the ferrous state. This was investigated by measuring the oxidation of ascorbic acid, in terms of oxygen uptake, in the presence of small

quantities of iron and varying quantities of added ATP. TABLE 6 shows quite clearly that ATP stimulates the oxidation of ascorbic acid as catalyzed by small quantities of ionic iron or of iron bound to serum. Furthermore, the optimal reaction velocity occurs at a molar ratio of ATP:ascorbic acid of 2:1, the same as was obtained for the incorporation reaction. The activity of ATP is specific

TABLE 4*
PROPERTIES OF FACTOR, IN ADDITION TO ATP, REQUIRED FOR SERUM Fe^{59}
INCORPORATION INTO FERRITIN†

Treatment of supernate	Ferritin iron specific activity (cpm/ μg . iron)	
	Without ATP	With ATP
Untreated	6.8	19.3
Heated to 100° C.	6.8	11.2
Dialyzed	3.2	3.3
Heated supernate + ascorbic acid oxidase	0.0	0.0

* Reproduced by permission from *The Journal of Biological Chemistry*.¹

† Each flask contained horse spleen ferritin with KCl-KHCO_3 solution and quantities of supernate, prepared by the centrifugation of a rat liver homogenate at 9000 g for 1 hour, equivalent to 0.1 gm. wet weight of liver. For treatment with ascorbic acid oxidase, the supernate was adjusted to pH 5.6, 116 units of enzyme added, and the mixture incubated for 15 min. at room temperature. The pH was then adjusted to 7.4, and the activity tested in the presence and in the absence of added ATP.

TABLE 5*
OPTIMAL RATIO OF ATP:ASCORBIC ACID FOR SERUM Fe^{59} INCORPORATION
INTO RAT FERRITIN†

Additions	Ferritin iron specific activity (cpm/ μg . iron)
None	0.7
20 ATP	3.4
20 ATP + 1 ascorbate	33.8
20 ATP + 5 ascorbate	50.2
20 ATP + 10 ascorbate	54.0
20 ATP + 20 ascorbate	52.0

* Reproduced by permission from *The Journal of Biological Chemistry*.¹

† Rat liver was the source of the partially purified ferritin. Serum Fe^{59} (50,000 cpm) was added to each flask which was incubated for 1 hour at 37° C. Concentrations of ATP and ascorbic acid are expressed as μmoles .

for iron; it inhibits the oxidation of ascorbic acid, which is catalyzed by cobalt or copper ions. These results suggest that ATP, ascorbic acid, and iron (ionic or transferrin-bound) form an activated complex that greatly enhances the flow of electrons, leading to an active reduction of ferric to ferrous iron.

As measured by the incorporation of serum-bound Fe^{59} into ferritin, ATP can be replaced by a number of biochemically important compounds, all containing a terminal pyrophosphate linkage, although ATP is most active on an equimolar basis (TABLE 7). Furthermore thus far, ascorbic acid can be re-

acted by only two compounds, each possessing an enediol grouping. These are glucoascorbic acid and dihydroxymaleic acid (FIGURE 1), which are equal in activity to ascorbic acid. Dihydroxy phenols with adjacent hydroxyl

TABLE 6*

EFFECTS OF ATP AND EDTA ON METAL-ION-CATALYZED OXIDATION OF ASCORBIC ACID†

Metal ion	Additions	QO ₂	Metal ion	Additions	QO ₂
None	Ascorbate	207	Serum‡	Ascorbate	194
+++	Ascorbate	326	Serum‡	Ascorbate + ATP	566
+++	6 Ascorbate + 1 ATP	635	Serum‡	Ascorbate + EDTA	306
+++	2 Ascorbate + 1 ATP	822			
+++	1 Ascorbate + 1 ATP	844	Cu ⁺⁺	Ascorbate	935
+++	1 Ascorbate + 2 ATP	955	Cu ⁺⁺	Ascorbate + ATP	536
+++	1 Ascorbate + 3 ATP	752	Cu ⁺⁺	Ascorbate + EDTA	306
+++	1 Ascorbate + 2 EDTA	925	Co ⁺⁺	Ascorbate	235
			Co ⁺⁺	Ascorbate + ATP	66
			Co ⁺⁺	Ascorbate + EDTA	146

* Reproduced by permission from *The Journal of Biological Chemistry*.¹

† Each Warburg flask contained 0.3 μ mole of metal ion, 120 μ moles of neutralized ascorbic acid, 0.8 ml. of 0.1 M phosphate buffer, pH 7.4, and ATP or EDTA as neutral salts in a total volume of 3.8 ml. The center well contained 0.2 ml. of 20 per cent KOH for carbon dioxide absorption. QO₂ values are calculated as μ l. of oxygen per hour from the observed oxygen uptake during the first 15 min. of the reaction. Flasks were incubated with shaking in air at 37° C. Values for ascorbic acid and ATP refer to μ moles; where no values are shown they are molar ratios of ascorbic acid to ATP or EDTA of 1:2.

‡ The quantity of rat serum used in this experiment contained 0.03 μ mole of bound iron

TABLE 7*

SPECIFICITY OF ATP FOR INCORPORATION REACTION†

Additions	Specific activity of ferritin iron (cpm/ μ g. iron)	Additions	Specific activity of ferritin iron (cpm/ μ g. iron)
None	2.2	Ascorbate + ADP	35.7
Ascorbate	5.6	Ascorbate + CTP¶	32.3
ATP	6.1	Ascorbate + PO ₄ ⁼	6.1
Ascorbate + ATP	69.5	Ascorbate + AMP**	5.6
Ascorbate + GTP‡	43.2	Ascorbate + DPN	5.4
Ascorbate + PP§	38.8	Ascorbate + TPN	5.4

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Each flask contained partially purified rat liver ferritin, together with Tris buffer, serum-bound Fe⁵⁹, 10 μ moles of ascorbic acid and 20 μ moles of the various phosphates tested.

GTP = Guanosine triphosphate.

PP = Inorganic pyrophosphate.

ADP = Adenosine diphosphate.

CTP = Cytidine triphosphate.

* AMP = Adenosine monophosphate.

are completely inactive. These results suggest that the complex formed on the iron atom involves the pyrophosphate linkage as well as the enediol oxygen atom of ascorbic acid.

Several years ago we observed a reaction that has many similarities with the 2-ascorbic acid reaction described above.⁵ We found that ethylenediamine-

tetraacetic acid (EDTA) markedly stimulated the iron-catalyzed oxidation of adrenaline and other dihydroxy phenols. The results shown in TABLE 6 demonstrate the specificity of iron in this reaction as well as the stimulating action of EDTA on the iron-catalyzed oxidation of ascorbic acid. In the case of ascorbic acid oxidation, EDTA and ATP have the same effect. However, unlike ATP, EDTA is not capable of bringing about the incorporation of serum-bound Fe^{59} into ferritin. This latter result suggests that the ATP-iron complex

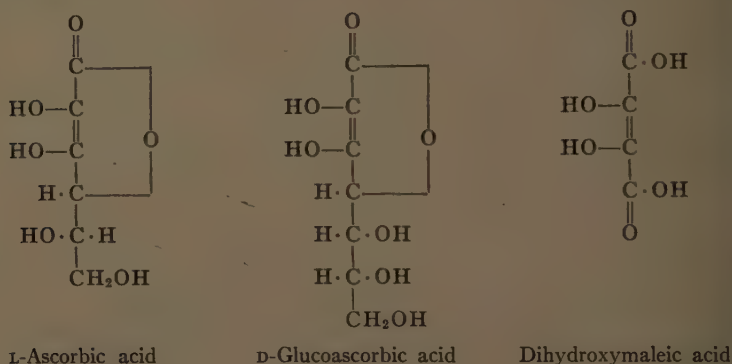


FIGURE 1.

TABLE 8
EFFECT OF ASCORBIC ACID DEFICIENCY ON SERUM IRON INCORPORATION INTO GUINEA PIG FERRITIN

	Controls*	Deficient
Liver		
Ferritin Fe^{59} (per cent of dose)	1.6 ± 0.3	$0.4 \pm 0.02\dagger$
Ferritin iron ($\mu\text{g.}$)	130 ± 25	$49 \pm 8\dagger$
Spleen		
Ferritin Fe^{59} (per cent of dose)	0.28 ± 0.08	$0.12 \pm 0.02\dagger$
Ferritin iron ($\mu\text{g.}$)	64 ± 15	$31 \pm 3\dagger$

* Control animals: 2 male and 3 female, average body weight 342 gm. (235-414). Deficient animals: 3 male and 3 female, average body weight 264 gm. (225-313). Results are expressed as the mean \pm the standard deviation.

† Significance of difference from control calculated from Student's test. $P < 0.001$.

‡ $P < 0.005$.

is susceptible of chemical alteration, thus liberating its iron for ferritin incorporation, whereas the EDTA-iron complex is not thus susceptible. The fact that EDTA is also active in catalyzing the ascorbic acid oxidation reaction suggests that the activated complex that carries out a rapid electron transfer does not depend on phosphate groupings as such.

Finally, we know something about the site at which iron is incorporated initially into the ferritin molecule. This site contains or is very near free sulfhydryl groups, since treatment of ferritin with compounds such as *p*-chloromercuribenzoate results in an inhibition of the incorporation reaction.

Although our data do not yet afford a reasonable explanation for the activity

the ATP-iron-ascorbic acid complex as an electron transfer system, the experimental results that have been presented suggest a good explanation for the release of tightly bound iron of transferrin and its incorporation into the ferritin molecule. It is quite likely that after its release from transferrin and entrance into the cell in the form of an ATP-Fe⁺⁺ complex, this form of iron may then be used not only for insertion into ferritin but also for the synthesis of other cellular iron complexes.

It is of some interest to note in connection with the problem of ascorbic acid oxidase activity that the ATP-iron complex may be considered as an artificial ascorbic acid oxidase, enhancing the activity of hydrated ionic iron. In terms of the naturally occurring ascorbic acid oxidase, the complexing site for copper may also involve a grouping that enhances its ability for ascorbic acid oxidation above that of hydrated ionic copper. This grouping, unlike ATP or EDTA, could form a specific complex with copper. A search for compounds capable of complexing copper and enhancing its activity as an ascorbic acid-oxidizing agent might suggest the nature of the site in ascorbic acid oxidase that binds the copper.

Our results present a new function for ascorbic acid, the reduction and release of ferric iron from its tight linkage to the plasma protein, transferrin, and its subsequent incorporation into tissue ferritin. Because of the requirement for ATP, the transfer of plasma-bound iron to the tissue and its incorporation into ferritin requires energy made available during the aerobic oxidation of foodstuff for the synthesis of ATP. Recent studies in our laboratory have confirmed the participation of ATP in the incorporation reaction in the intact rat. TABLE 8 illustrates the marked inhibition of serum iron incorporation into ferritin of the liver and spleen of the ascorbic acid-deficient guinea pig. Since man is dependent on his diet for ascorbic acid, this vitamin may be said to play an important role in one phase of iron metabolism: the movement of plasma iron to storage depots in the tissues.

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HUMAN REQUIREMENTS FOR VITAMIN C AND ITS USE IN CLINICAL MEDICINE

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Human requirements for vitamin C have been investigated extensively. The minimal quantity that will prevent the development of scurvy has been established with reasonable certainty, but the desirable intake for the maintenance of health remains unknown and is a subject of considerable controversy. The amount of ascorbic acid that has been found to prevent scurvy is approximately 10 mg. a day or somewhat less. The first information in this regard stems from the observations of Lind in 1753 who showed that scurvy could be prevented by a daily allowance of 25 to 30 ml. of lemon juice. According to present knowledge, this quantity of lemon juice should furnish 10 to 15 mg. of ascorbic acid. Early experiences indicated that the substitution of lime juice for lemon juice was not an effective preventive measure.¹ Lime juice contains about one-fourth as much vitamin C as does lemon juice.

Infantile scurvy is a disease of modern times that was noted with increasing frequency as formulas of cow's milk replaced breast feeding. In infants who received raw diluted cow's milk, mild symptoms of scurvy were not rare. In these instances, it was estimated by Uhl² that the infant received 3 to 6 mg. of ascorbic acid daily; hence the minimum requirement must be greater than this. Van Eekelen³ reported that infants who received raw cow's milk containing 2 mg. of ascorbic acid per 100 ml. or boiled cow's milk containing 1.1 mg./100 ml., or a total intake of about 7 mg. of ascorbic acid daily, do not get scurvy. Vitamin C deficiency is extremely rare in infants who are breast-fed. The quantity of ascorbic acid in human milk fluctuates according to the maternal diet. In a study in the Netherlands,³ it was found that mother's milk contained between 0.8 and 3.5 mg./100 ml. (average 1.8 mg./100 ml.) during the season when least ascorbic acid was eaten, whereas the content ranged between 4.1 and 6.8 mg./100 ml. when more ascorbic acid was available. It is generally stated that if the mother's diet is reasonably good the milk will contain 4 to 8 mg./100 ml. In such instances, breast-fed infants receive a daily allowance of at least 20 mg. of ascorbic acid and often as much as 40 to 50 mg. daily. Such infants do not develop scurvy. Hamil and his associates⁴ in a study of 427 infants found that 10 mg. of vitamin C daily would protect from scurvy. During the course of their study, 21 infants developed mild evidence of the disease according to the criteria of Park *et al.*⁵⁴ Fourteen of these 21 infants received an average of 9 mg. of ascorbic acid daily; 7 received less than this amount.

Ingalls⁵ showed progressive exhaustion of vitamin C reserves with increasing age in premature infants who died before 4 months of age and who had received only pasteurized mother's milk. Histologic changes were present in the bone and costochondral junctions although no signs of scurvy were apparent during life.

Plasma ascorbic acid levels in healthy breast-fed infants have been reported

range between 0.5 and 1.5 mg./100 ml., whereas levels in healthy infants and diluted cow's milk as the sole source of food averaged 0.25 mg./100 ml.² The administration of 20 mg. of ascorbic acid as a supplement, but not 10 mg., raised the plasma concentration of the latter infants to 0.56 to 0.76 mg./100 ml. The ascorbic acid supplement increased the dietary supply to 5 to 6 mg. of ascorbic acid per kilogram of body weight. In another study,⁶ it was shown that the concentration of ascorbic acid in plasma could be increased from 0.4 to 0.8 mg./100 ml. by administration of 20 mg. or more of ascorbic acid daily, and that 20 mg. was as satisfactory as 75 mg.

From these data it appears that approximately 10 mg. of ascorbic acid daily will prevent infantile scurvy. In infants fed cow's milk, a daily supplement of 20 mg. of vitamin C is required to bring plasma ascorbic acid concentration up to that observed in breast-fed infants.

The minimum requirement of vitamin C for prevention of scurvy in adults appears to be somewhat less than 10 mg. daily. An investigation by the British Medical Research Council in 1944-1946⁷ showed that daily administration of 10 mg. of ascorbic acid to healthy adults who received a diet essentially devoid of this vitamin, but otherwise adequate, prevented the appearance of scurvy over a period of 12 months. Furthermore, administration of 10 mg. of ascorbic acid a day to subjects who developed scurvy on the basal diet was sufficient to lead to healing of all signs of the disease in 10 to 14 weeks. In a study of Royal Canadian Air Force personnel,⁸ one group was maintained on a diet that furnished 8 mg. of ascorbic acid daily for 8 months. No signs of scurvy developed during this period.

Although scurvy may be prevented by 10 mg. or less of ascorbic acid daily, available evidence suggests that the desirable intake for maintenance of optimal health may be considerably greater than this. All animal species except primates and guinea pigs can synthesize vitamin C and have no dietary requirements. In these species a state of tissue saturation exists. Thus from a teleological standpoint, it may be argued that saturation of the tissues may be the desirable state in man. Studies in guinea pigs, a species that, like man, does not synthesize vitamin C, indicate that the quantity needed to prevent obvious signs of scurvy is considerably less than that necessary to ensure full potentialities of growth or to maintain normal function of odontoblasts.

In man certain studies indicate that more ascorbic acid is needed for the maintenance of healthy gums, for normal growth, for optimal reactions to physiologic and pathologic stress, and for provision of reasonable tissue stores for the prevention of scurvy. Much information has been accumulated as to the quantity of ascorbic acid needed for tissue saturation, but there is no direct proof that saturation will provide definite benefits. However, these data have a bearing on recommended dietary allowances of vitamin C and will be discussed in some detail. In several studies, vitamin C intake and the degree of saturation of the tissues have been related to physiologic response or physiologic change.

Methods that have been used to estimate the degree of saturation of the tissues with ascorbic acid have included determination of urinary excretion, measurement of levels of ascorbic acid in plasma, estimation of the ascorbic

acid content of the white cell-platelet layer of blood, and the response to "load" tests.

Lowry and his associates⁹ studied repletion of body stores of ascorbic acid in groups of young men who had been maintained for 8 months on daily intakes of 8 and 23 mg. of ascorbic acid. They found that the concentration of ascorbic acid in leukocytes paralleled ascorbic acid retention as measured by urinary secretion. These investigators calculated that the tissue deficit of the two groups of men who received 8 and 23 mg. of ascorbic acid daily was approximately the same, amounting to about 1800 mg. During the period on these low intakes, concentration of ascorbic acid in white cells had fallen from about 25 mg. per cent to 11.9 and 12.9 mg. per cent respectively; that is, there was a decrease of about 50 per cent. Accordingly it was estimated that total body stores of ascorbic acid were somewhat less than 4 gm. In a third group of men who were maintained on 78 mg. of ascorbic acid daily, the tissues were about 90 per cent saturated as compared to 50 to 60 per cent saturation in the other two groups. There appeared to be some handicap to the gums in the groups receiving 8 and 23 mg.⁸ These findings suggest that at some place between 60 and 90 per cent saturation, the gums may be affected by an inadequate supply of ascorbic acid.

In a large number of studies, particularly nutrition surveys, an attempt has been made to relate ascorbic acid intake to the condition of the gums. Both positive and negative results have been obtained. Some of these reports were reviewed previously.¹⁰ Several recent investigations are pertinent to the problem of desirable intake of ascorbic acid and will be mentioned here.

Morgan and her associates¹¹ found a slight relationship between the level of ascorbic acid in serum and the condition of the gums in a study of an aged population. Seventeen per cent of the subjects examined had gingivitis to some degree, and the greatest proportion of persons showing this lesion were in the group in which ascorbic acid concentration was below 0.3 mg. per cent.

Martin and her co-workers¹² in the Vanderbilt cooperative study on maternal and infant nutrition found a significant relationship between gingival findings and intake of vitamin C when groups with "consistently" low (<40 mg.), medium (40 to 79 mg.), and high (>80 mg.) intakes were compared on the basis of the number having gum findings on two or more physical examinations. Among women of parity two or less with "consistently" low vitamin C intakes 19 per cent had gum findings, whereas the corresponding percentages were 14 and 9 in the "consistently" medium and high intake groups respectively.

Data obtained in eight nutrition surveys conducted by the Interdepartmental Committee for Nutrition in National Defense¹³ showed a correlation between the measured dietary intake of vitamin C and clinical findings of bleeding gums or diffusely swollen, dusky, and friable gums ("scorbutic type"). The individual mess survey data showed a rather definite cutoff point at about 30 mg. of vitamin per day, below which an increased incidence of the lesions occurred. Plasma vitamin C levels were also correlated with the gingival lesions.

In an investigation some years ago, Linghorne and his associates¹⁴ found that when gingivitis was cleared up by local treatment, provision of 75 mg. of ascorbic acid had a delaying effect on the recurrence of signs of inflammation.

s compared with that observed with 10 mg. of ascorbic acid daily. Results indicated also that 75 mg. retarded recurrence of gingivitis to a greater degree than did 25 mg. The histological appearance of the gingival tissues did not resemble the pathologic changes in scurvy.

Pierce and his associates¹⁵ conducted a 3-year investigation of changes in the gum tissue in relation to ascorbic acid intake and to levels of ascorbic acid in serum and white cells. A control group received 47 to 60 mg. of ascorbic acid daily, while a test group was given a supplement that raised the intake to 40 to 412 mg. The supplement had no significant effect on pitting, swelling, hunting, recession, and abnormal color of the gums although the incidence of these findings was high in both groups. In another study, Ogilvie and Pett¹⁶ observed the effect of supplementation of the diet with 100 mg. of ascorbic acid daily on the gums of children. The initial food record showed a daily intake of 50 mg. of ascorbic acid per person. The children were studied for a period of 4 years, half of the group receiving a supplement and the remainder a placebo. Gingivitis developed as frequently in the supplemented as in the controlled subjects. Red, swollen gums, characteristic of gingivitis but not of scurvy, developed in about the same percentage of children in both groups.

The last two studies mentioned indicate that no benefit to the gums resulted from intakes of ascorbic acid greater than 50 mg. daily. The other investigations cited strongly suggest that an intake of about 30 mg. of ascorbic acid is necessary for the maintenance of healthy gums and that at least 60 per cent saturation of body tissues with ascorbic acid is desirable.

Lowry¹⁷ has reviewed relationships between ascorbic acid intake and concentrations of ascorbic acid in the plasma and white cells and urinary excretion of the vitamin. When plasma levels are above 0.4 mg. per cent, concentrations in white cells are usually above 20 mg. per cent; when plasma levels are less than 0.3 mg. per cent, white cell values are less than 20 mg. per cent. When the intake of ascorbic acid is about 100 mg. or more daily, the tissues and white cells are approximately saturated and will contain a total of about 50 mg. of ascorbic acid per kilogram of body weight, serum levels will be above 1.0 mg. per cent, and white cell levels above 25 mg. per cent. Urinary excretion will measure the intake; that is, there will be recovery of 60 to 80 per cent of an administered dose of the vitamin. When the ascorbic acid intake is 40 to 100 mg. daily, plasma levels are 0.4 to 1.0 mg. per cent, the tissues and white blood cells are saturated with ascorbic acid or only moderately below the ceiling, and the urinary excretion is 20 to 60 per cent of the intake. When the ascorbic acid intake is less than 40 mg. daily, tissue levels will fall reaching 50 per cent saturation with 10 to 15 mg. per day and, by extrapolation with the guinea pig, 25 per cent saturation when the intake is 5 to 7 mg. per day. In this range, plasma concentrations are not accurate, and the concentration in white cells must serve to determine the extent of deficiency.

Peters and his associates,¹⁸ in a report of the British Medical Research Council's investigation on vitamin C requirements, noted similar relationships between vitamin C intake and the concentration of ascorbic acid in serum and white cells. Blood levels were determined after periods of 47 to 336 days on a given level of intake. With an intake of 20 mg., the average plasma level was less than 0.1 mg. per cent and the white cell level 3.6 mg. per cent. When

the intake was 50 mg., the plasma level was 0.3 mg. per cent and the white cell level 8.6 mg. per cent. An intake of 70 mg. was associated with a plasma level of 0.5 mg. per cent and a white cell level of 10 mg. per cent; an intake of 100 mg. was associated with a plasma level of approximately 1 mg. per cent and a white cell concentration of 17 mg. per cent. These concentrations are somewhat lower than those reported by Lowry and his associates,^{9,17} presumably due to differences in methodology. In the British study, ascorbic acid concentration of white cells fell to less than 2 mg. per cent prior to the appearance of scurvy. In this study also, tests of physical fatigue left some doubt as to whether 10 mg. was an optimal dose of ascorbic acid. Although results were not conclusive, statistical analyses revealed small differences in favor of the group receiving 70 mg. as against the group receiving 10 mg. of ascorbic acid daily.

Morse and her associates¹⁹ investigated the amount of ascorbic acid required for tissue saturation. In one series of experiments, saturation was reached in 5 weeks on an intake of 83 mg. of ascorbic acid daily; white cell ascorbic acid was 34 mg. per cent and serum concentration was 1.4 mg. per cent. On an intake of 58 mg. daily, white cell and serum ascorbic acid levels were 26.7 mg. and 0.7 mg. per cent respectively. In another study²⁰ an intake of 57 mg. of ascorbic acid a day resulted in saturation of the white cells in both younger and older women. Steele and her associates²¹ measured ascorbic acid in serum and white cells in subjects receiving controlled low intakes of the vitamin. After a period of 38 to 42 days on a 10-mg. intake, the diet was continued and the intake increased to 20, 30, and 40 mg. daily for 14 days. There was no significant increase in ascorbic acid concentration in serum or white cells when the intake was changed to 20 or 30 mg. daily, but there was a significant increase when 40 mg. of ascorbic acid was given. In earlier studies Kellie and Zilva²² found in one subject that when 30 mg. of ascorbic acid was ingested daily, almost none was excreted in the urine, suggesting complete utilization. When the dietary intake was 100 mg., approximately 50 mg. was metabolized. When the intake was 50 mg., about 40 mg. was metabolized.

Haines and her associates²³ studied tissue reserves of ascorbic acid in normal adults on 3 levels of intake. After a preliminary period of saturation, subjects were put on diets that furnished 33, 50 to 53, and 70 mg. daily of ascorbic acid for 6 weeks. The response to a 400-mg. test dose of ascorbic acid was then determined. Depletion of tissue stores was noted on all intakes, being especially marked on that of 33 mg. daily. Although the 70-mg. intake did not maintain tissue saturation, a plateau was reached. Lamden and his associates²⁴ found that with an ascorbic acid intake of about 60 mg. daily, the average white cell ascorbic acid level was 28 mg. per cent and the serum level 1 mg. per cent. Supplementation of a test group with large amounts of ascorbic acid, resulting in a total intake of 340 to 412 mg. daily, caused little increase in white cell levels. These ranged from 32 to 36 mg. per cent while serum concentrations varied from 1.5 to 2 mg. per cent. Dodds and her associates²⁵ studied 24 college women on intakes of 50, 75, and 100 mg. of ascorbic acid for periods of 12 days each. Retention was calculated from experimental data and from estimated "equilibrium values." Their findings showed a utilization of close to 1.0 mg. of ascorbic acid per kilogram of body weight daily. The amount of vitamin

in the basal diet was 6.9 mg. Utilization was essentially the same on all levels of intake.

Belser and her associates²⁶ reported that 1.0 to 1.7 mg. of ascorbic acid per gram of body weight was required to maintain tissue saturation. Dodds and MacLeod²⁷ found that an intake of 1.0 mg./kg. increased plasma ascorbic acid in 12 subjects and produced saturation in 3 of them. Corresponding data of Todhunter and Robbins²⁸ indicated that 1.6, 1.7, and slightly more than 1.8 mg./kg. were required for saturation of 3 subjects.

The findings discussed above suggest that an intake of less than 40 mg. of ascorbic acid daily is associated with rapid depletion of tissue stores and that an intake of 60 to 100 mg. is needed for saturation or near saturation of the tissues. Findings of similar nature have been used in the estimation of recommended dietary allowances of several of the B complex vitamins.

Recent studies with labeled ascorbic acid show that requirements differ among animal species. The guinea pig was found to require an average of 10 mg./kg. of body weight per day to maintain a body pool of 54 mg./kg. The guinea pig required 26 mg./kg. daily to maintain a body pool of 107 mg./kg. Studies of Hellman and Burns²⁹ in 3 human subjects using L-ascorbic acid-1-C¹⁴ showed a body pool of ascorbic acid of 19 to 26 mg./kg. (average 20 mg./kg.) and a turnover rate of 0.66 to 1.4 mg./kg. per day (average 1 mg./kg.). These findings may explain the lower requirements for ascorbic acid in man as compared with the guinea pig.

Crandon and his associates³⁰ studied white-cell and plasma ascorbic acid concentrations in 561 surgical patients. They stated that the more-or-less predictable relationships between serum and tissue ascorbic acid seen in acute deprivation experiments in otherwise normal persons are not found in the sick patient. Data obtained in these studies indicated that plasma concentrations less than 0.2 mg./100 ml. are suggestive of serious ascorbic acid deficiency and that healing will take place if white-cell levels are above 8 mg./100 gm. if no wound infection or local stress occurs. Patients with deficient plasma ascorbic acid and white-cell ascorbic acid concentrations have a relatively poor prognosis and a high incidence of evisceration, incisional hernia, and draining wound healing problems postoperatively. Crandon and his associates³¹ believe that the maintenance need for ascorbic acid is markedly increased in some surgical patients and that requirement varies with the degree of inflammatory response. Findings reported by these workers suggest but do not prove this to be the case. The difficulties in control of experiments in acutely ill patients are obvious and require further investigation. The amount of ascorbic acid that will maintain tissue levels adequate for normal wound healing in clinical situations in which infection and other stress factors may be operating remains unknown. Further studies in this area with the best possible control of variables could add much to current knowledge of desirable ascorbic acid intake.

Very few data are available relative to the minimum requirement of ascorbic acid in children or the amounts needed for the maintenance of tissue saturation. In a study of 3 children age 3 to 5 years,⁶ the amount of ascorbic acid required for saturation of the tissues varied from 6.4 to 7.5 mg./kg. of body weight per day or a total of 117 to 121 mg. of ascorbic acid daily. In a study of school children 8 to 13 years of age living in temperate climates, Roberts and Roberts³²

found that 105 to 125 mg. of ascorbic acid per day was needed to produce tissue saturation but that plasma levels above 0.7 mg./100 ml. could be maintained on daily intakes of 65 to 75 mg. Bessey and White³³ showed that in children 5 to 13 years of age 45 mg. of ascorbic acid per day gave maximum plasma levels of about 1 mg./100 ml. Nicol³⁴ studied two groups of Nigerian children aged 9 to 15 years who were paired as to age, sex, and height. The customary diet supplied about 16 mg. of ascorbic acid daily for 10 months of the year and about 170 mg. during the remaining 2 months. One group received a supplement of ascorbic acid throughout the year while the control group received placebo tablets that resembled ascorbic acid. The average yearly supplement was 33 ± 4 mg. The average gain in height of the children in the group treated with ascorbic acid was significantly greater than that of the untreated group. Gain in body weight was unaffected by the supplement of vitamin C. Nicol concluded that the children's average daily intake of ascorbic acid was insufficient to maintain maximum growth rate. He found no significant reduction in the incidence of disease that could be attributed to ascorbic acid supplementation.

Vitamin C requirement appears to be increased during pregnancy and lactation. This might be anticipated since vitamin C plays an important role in growth processes and is found in abundance in active and growing tissues. It has been estimated that for milk production of 800 cc. per day, containing mg. of ascorbic acid per 100 ml., the added requirement will be 50 mg.⁶

The fetal needs must be met from the stores of the mother and the maternal diet. Placental tissue contains relatively high concentrations of the vitamin and the levels of ascorbic acid in the plasma of the infant are two to four times those in maternal plasma. This finding supports the theory that the placenta exerts selective control of vitamin C passage and may act as a barrier to reentrance of ascorbic acid into the maternal circulation. It is generally recognized that fetal needs take priority over maternal needs for vitamin C and that the fetus may build up a substantial reserve at the expense of the mother. The importance of vitamin C "nutriture" during pregnancy has been shown by Smith and his associates,³⁵ who found a statistically significant relationship between the amount of ascorbic acid in the fetal liver and the amount of the vitamin in the maternal diet.

Some investigators have reported no significant changes in serum ascorbic acid levels as pregnancy progresses, whereas others have noted a slight downward trend in successive trimesters with a decrease after delivery. Macy and her associates³⁶ found decreases in plasma ascorbic acid in both Negro and white women between the first and third trimesters of pregnancy and a further decrease postpartally. The lower postpartum concentrations may reflect a decrease in food intake, an increased need for vitamin C for wound healing, an increased requirement for milk production, and perhaps also for endocrine readjustment. In the Vanderbilt cooperative study of maternal and infant nutrition,¹² vitamin C intakes and serum levels were determined in 2129 pregnant women. In general, serum concentration decreased during pregnancy except in the group with a high level of intake. A daily intake of 80 to 100 mg. of ascorbic acid during pregnancy supported high levels in the serum. After delivery there was a further decrease in serum ascorbic acid concentra-

n. The serum of nonlactating women averaged 0.7 mg./100 ml. during the puerperium when the intake during pregnancy was 100 mg. a day or more. Serum concentrations of lactating mothers did not average more than 0.3 mg./100 ml. even on intakes exceeding 120 mg. daily. In an analysis of relationships between the health of the mother and baby and ascorbic acid nutriture, it was found that there was some relationship, although not a strong one, between ascorbic acid intake and serum levels during pregnancy and hematologic findings, gingivitis, premature separation of the placenta, premature birth, puerperal fever. The increased incidence of premature birth was limited to the lowest intake levels or lowest serum concentrations. Martin *et al.*¹² concluded that ascorbic nutriture was at most a contributory factor in these conditions.

In view of the data summarized above, what should be the recommendation for the desirable intake of ascorbic acid for the maintenance of health? The League of Nations⁶⁵ technical commission on nutrition in 1938 suggested 30 mg. of ascorbic acid daily as ensuring a reasonable margin of safety. This amount was approved by the Medical Research Council of Great Britain as a result of their studies of vitamin C deprivation. From the data recorded above, 30 mg. daily cannot be expected to furnish large tissue reserves, and less than this amount might not maintain normal gingival tissue. The Recommended Dietary Allowances of the Food and Nutrition Board of the National Academy of Sciences-National Research Council of the United States are considerably higher than this, for example, 70 to 75 mg. daily for adults. As Harris³⁷ indicated in his summary of the proceedings of the Lind Bicentenary Symposium, "One thing that would help more than anything else in settling this controversy about requirements would be to have new functional tests developed to demonstrate the effect of minor degrees of deficiency." Such tests might involve some kind of local stress situation in view of the observations of Crandon and his associates.³⁰ When more is learned about the role of vitamin C in metabolism, it may be possible to devise procedures that will indicate early functional impairment. The known functions of vitamin C include a reducing action that enables this vitamin to participate in or to stimulate a large number of chemical reactions. However, this function appears to be largely nonspecific and other reducing agents can often replace the vitamin. Two of these reactions that have been studied in man are the influence of ascorbic acid on the conversion of folic acid to folinic acid and the role of this vitamin in the metabolism of tyrosine and related substances. Abnormalities of folic acid and tyrosine metabolism are not encountered until vitamin C deficiency is well advanced.³⁸ Ascorbic acid has been found to be related to adrenal function and to the metabolism of cholesterol, but its exact metabolic role remains to be elucidated. Vitamin C is needed for the normal functional activity of protein-formative cells such as odontoblasts, ameloblasts, cementoblasts, and osteoblasts; and in vitamin C deficiency the production of collagen is impaired. Yet none of these functions can be tested readily in human subjects either clinically or biochemically. In view of the many potential roles of vitamin C in metabolism and the evidence suggesting that vitamin C is useful in amounts far in excess of those necessary for the prevention of scurvy, it would seem wise to provide a daily allowance of ascorbic acid that would ensure good tissue

stores of the vitamin. It is hazardous to suggest an exact level of desirable intake. Nevertheless, from the data summarized in this review, it would appear that infants might well receive 20 mg. or more daily. As for adults, 4 mg. daily will support moderate tissue stores of ascorbic acid, and 60 to 100 mg. will provide tissue levels approaching saturation.

The Use of Vitamin C in Clinical Medicine

The primary use of ascorbic acid in clinical medicine is in the prevention and treatment of scurvy. As indicated in the preceding section, scurvy can be prevented by the administration of 10 mg. of vitamin C daily. However, it is usual pediatric practice to administer considerably more than this, at least 20 mg. and often 2 or 3 times this amount, beginning in the first weeks of life. This is usually given in the form of orange juice. The treatment of scurvy requires larger doses to refill depleted tissue stores. In infants, 25 mg. 4 times daily will suffice, while in adults 100 mg. 5 or 6 times daily will result in rapid restoration to normal. The vitamin is usually given in frequent small doses to avoid exceeding the renal threshold, which is in the neighborhood of 1.4 mg. per 100 ml. With sufficient dosage it is possible to replace tissue stores within a few days. There is no danger of toxicity from vitamin C in amounts much larger than those needed for rehabilitation or maintenance because of the rapid excretion of the excess by the kidney.

Adequate supplies of vitamin C are necessary for the normal healing process. There is general agreement that vitamin C is required for the maturation of collagen and adequate capillary invasion of healing wounds. Abt and his associates³⁹ in studies of guinea pigs found that ascorbic acid is essential for the production of connective tissue for immediate postoperative healing and also for maintenance of connective tissue in previously formed scar tissue. They also showed a direct relationship between the amount of vitamin C in the diet and the strength of the healing wound, as well as between the dietary level of ascorbic acid and its concentration in scar tissues, in the tissues of other organs, and in the blood. Abt *et al.* demonstrated that ascorbic acid accumulates in scar tissue immediately following wounding and persists for long periods of time. The highest concentration in the wound is in the connective tissue. In human subjects, numerous reports have indicated a decrease in plasma ascorbic acid following trauma or surgical operations. This appears to be due to a shift of ascorbic acid from the serum to the site of wounding. Many clinical reports have indicated that low ascorbic acid levels are found almost routinely in patients with wound disruption.⁴⁰ Wolfer and his co-workers⁴¹ reported that in human subjects with prolonged ascorbic acid depletion there was a 50 per cent diminution in the tensile strength of wounds. The high incidence of wound infection in these patients suggested a need for ascorbic acid in the tissues for maximum resistance to infection.

Studies of experimental vitamin C deficiency in man indicate that deficiency must be quite severe before wound healing is inadequate. The studies of Crandon and his associates^{30,31} mentioned previously suggest that findings in sick patients differ considerably from those in healthy subjects deprived of ascorbic acid. They reported a marked increase in requirement in some surgical patients, which seemed to vary with the degree of inflammatory response.

is common practice at the present time to treat patients who require surgery with large doses of ascorbic acid pre- and postoperatively. Unfortunately, there are no clinical reports on wound healing and the postoperative course of patients who were alternately treated with large doses of vitamin C and a vitamin supplement.

Ascorbic acid has a role in hematopoiesis and is useful in the prevention and therapy of certain types of anemia. In scurvy, anemia is a common finding and, in advanced cases, it may be of the megaloblastic type. Several investigators have shown that the anemia of scurvy will respond to ascorbic acid alone.⁴² Others have found the anemia in scorbutic subjects to be associated with deficiency of iron and to respond to this medication. Megaloblastic anemia of infancy, reported not infrequently in this country some years ago, appears to be associated with ascorbic acid deficiency. This anemia responds to treatment with folic acid. The addition of ascorbic acid to certain proprietary milk formulas has resulted in virtual disappearance of this type of anemia.⁴³ Of interest in this connection is the finding that folic acid deficiency in guinea pigs may lead to the appearance of megaloblasts in the bone marrow.⁴⁴ The incidence of megaloblastosis is increased by simultaneous deficiency of ascorbic acid. May and his associates⁴⁵ produced megaloblastic anemia in monkeys on diets deficient in ascorbic acid and low in folic acid. They indicated that conversion of folic acid to folinic acid *in vivo* may be less efficient in scurvy but that ascorbic acid is not necessary for this conversion to take place. Other phases of the metabolism of folic acid compounds that were studied were found to be normal. Ascorbic acid deficiency was not accompanied by a marked deficiency of folic acid compounds until signs of scurvy had become well advanced. These investigators stated their belief that the severe deficiency of folic acid compounds that occurred regularly as a complication of scurvy in monkeys fed milk diets was probably due to nonspecific factors operating in scurvy and that the net effect of scurvy was to cause increased requirements for folic acid. In other reports, May and his associates⁴⁶ indicated that the megaloblastic anemia produced in monkeys receiving diets low in ascorbic acid and folic acids will respond to folic acid and also to ascorbic acid, although in the latter instance the megaloblastosis disappeared slowly. This was followed, however, by a rapid rise in hemoglobin.

The role of ascorbic acid in the metabolism of folic acid has not been completely elucidated. Ascorbic acid augments the conversion of folic acid to folinic acid, probably by its action as a reducing agent. The administration of folic acid in conjunction with ascorbic acid to human subjects increases the urinary excretion of folinic acid to about three times that observed when folic acid is administered alone.⁴⁷ In patients with scurvy, administration of a large dose of folic acid is followed by excretion of subnormal amounts of folinic acid. Jandl and his associates⁴⁸ reported a reticulocyte response to small doses of folic acid in two scorbutic subjects with macrocytic anemia. A second reticulocytosis followed administration of ascorbic acid.

Hueller and Will⁴⁹ have suggested that ascorbic acid has an indirect action on folic acid metabolism in at least two different sites. They also indicated a relationship between vitamin B₁₂ and ascorbic acid metabolism. They pointed out that ascorbic acid metabolism may be altered in patients with megaloblastic

anemia. Plasma ascorbic acid concentrations were found to be subnormal in patients with macrocytic anemia as compared with subjects with similar dietary background but without anemia. Vitamin C was capable of producing reticulocytosis and some improvement in anemia in several of these patients. Boscott and Cooke⁵⁰ reported a disturbance of ascorbic acid metabolism in nontropical sprue. This was evidenced by the persistent excretion of parahydroxyphenylacetic acid in the urine. Patients with pernicious anemia also show an abnormal excretion of tyrosine derivatives in the urine, and similar findings are characteristic of scurvy.

Moore⁵¹ found that ascorbic acid in relatively large amounts, given either in crystalline form or as fruit juice, increased the assimilation of food iron. This increase was greater in patients with iron deficiency than in normal subjects. It was demonstrated also that reducing substances such as cysteine have a similar action while other organic acids are without effect. Gortner and Bradley⁵² reported a favorable effect of ascorbic acid on iron absorption in the treatment of nutritional anemia in infants and children. It was postulated that the vitamin improved iron utilization as well as absorption. Greenberg and his associates⁵³ in studies with rats observed an interrelationship between ascorbic acid and vitamin E in iron metabolism. Convincing evidence was presented that a combination of ascorbic acid and vitamin E enhanced the synthesis and maintenance of hemoglobin more than did either vitamin alone. Whether the increased efficiency of conversion of iron to hemoglobin might be due to increased absorption of iron or increased efficiency of utilization by the tissues could not be determined from the experiments.

Numerous investigations in experimental animals have indicated a relationship between ascorbic acid, stress situations, and the function of the adrenal glands. Recently Kark⁵⁴ summarized some of the pertinent findings in this area. He quoted Lund and Levenson and their colleagues as finding that both the plasma levels and urinary output of ascorbic acid dropped to low values in the postoperative period, despite treatment with very high doses of ascorbic acid. These investigators explained the above findings on the basis of increased utilization of vitamin C. Kark noted that the modern view inclines to the belief that ascorbic acid requirements for man are increased as a result of a variety of stressful situations such as burns, severe injuries, operations, infections, and rheumatic diseases. Studies in rats showed that the ascorbic acid and cholesterol content of the adrenal glands decreased when the animals were stressed or when their adrenal cortices were stimulated with corticotropin. These findings led Levenson and Lund to postulate that the increased demand for and utilization of ascorbic acid were related to increased consumption of the vitamin by the adrenal gland.

Many physicians believe that large amounts of ascorbic acid are consumed by the adrenal glands during illness in the synthesis of cortical hormones from cholesterol. Conversely, the thesis that adrenal activity will be sharply reduced when ascorbic acid supplies are curtailed is held by many. These concepts have been found to be erroneous. As Kark pointed out, interrelationships between ascorbic acid metabolism and adrenocortical activity need reorientation, at least with regard to the guinea pig, monkey, and man, who do not synthesize ascorbic acid in the adrenal glands. Observations of adrenal

activity in patients with scurvy indicate that the adrenal cortex functions normally in the face of gross vitamin C deficiency. Excretion of 17-ketosteroids is normal, and the adrenal glands respond normally to stimulation with corticotropin. In monkeys also, the adrenal cortex functions normally during scurvy and responds in a normal manner to repeated stimuli with corticotropin. It seems obvious, therefore, that the adrenal glands cannot consume much ascorbic acid in the synthesis of cortical hormones. In Cushing's syndrome in man, in which there is hyperactivity of the adrenal glands, ascorbic acid levels in the blood are normal, and there is no evidence of scurvy. Since the activity of the adrenal cortex of man does not seem to require ascorbic acid for synthesis of cortical hormones, the disappearance and retention of ascorbic acid in the traumatized patients studied by Levenson and Lund may be explained most satisfactorily by the finding mentioned previously: that ascorbic acid is mobilized from the tissues and organs of the body and selectively concentrated in the traumatized area. Thus plasma and urinary levels for vitamin C might be expected to be decreased. These findings do not rule out increased requirements for vitamin C during stress.

Recently Holley and McLester⁵⁵ presented the cases of two patients with rheumatoid arthritis who had been treated for long periods with corticotropin and developed signs of scurvy. In both instances the hemorrhagic manifestations receded dramatically with massive doses of ascorbic acid. Supplements of this vitamin prevented reappearance of symptoms during subsequent courses of corticotropin therapy. Thus it is possible that prolonged adrenal stimulation may deplete adrenal and total body stores of ascorbic acid. Chamberlain and Addison⁵⁶ reported that they had seen a number of cases of scurvy following bilateral adrenalectomy and oophorectomy for carcinoma of the breast. They described two cases in which purpuric spots developed about 10 days after operation and, in each instance, a vitamin C saturation test indicated deficiency. Development of scurvy within 10 days after operation is certainly remarkable, and further investigation seems desirable.

While numerous findings suggest that ascorbic acid is involved in some way in the production or secretion of adrenocortical hormones, the mechanism of action is not clear. The concept that ascorbic acid constitutes a limiting factor in the synthesis of corticosteroids cannot be reconciled with the continued formation of these hormones in depleted animals. There is some evidence that large doses of ascorbic acid improve the utilization of corticoid hormones and prolong their action by delaying breakdown and excretion. The many uncertainties make it obvious that additional study is needed to determine the value of therapy with ascorbic acid in stress situations in man.

In early studies of the use of ascorbic acid in clinical medicine, low values for the vitamin were reported in plasma and urine during the course of a wide variety of infections. These included mild coryza, acute pharyngitis, persistent chronic nasal sinusitis, chronic otitis media, acute respiratory infections, active poliomyelitis, rheumatic fever, and rheumatoid arthritis. A favorable response to treatment with ascorbic acid was reported in pneumonia, pertussis, and rheumatic fever. Ascorbic acid was considered to be of assistance in the healing of tuberculous lesions. In numerous early reports of scurvy,⁵⁸ emphasis was placed on the precipitation of scorbutic symptoms by infection and also on

the high susceptibility to infection shown by patients with scurvy. Infection occurring in association with active scurvy were said to be more severe and to carry a grave prognosis. These findings were interpreted as indicative of an increased requirement for vitamin C during infections. In the light of recent information, the decrease in plasma and urinary ascorbic acid in association with infections may be due to a shift of ascorbic acid to the tissues affected by the infectious process.

A high incidence of vitamin C deficiency has been found in patients with pellagra, chronic alcoholism, and peptic ulcer (when treated with the Sippy diet). In these situations intake of ascorbic acid was undoubtedly low. Subjects receiving large doses of alkali or patients with achlorhydria have been said to develop ascorbic acid deficiency frequently, presumably due to destruction of the vitamin in the intestinal tract. Diarrheal diseases have been found to interfere with absorption of ascorbic acid and to lead to deficiency of this vitamin. Thyrotoxicosis has been reported to increase the amount of ascorbic acid utilized.⁵⁷

In other early studies vitamin C was used in the prevention of toxicity from certain drugs such as neoarsphenamine. Administration of ascorbic acid made it possible to treat patients who had shown intolerance to the drug in question. Ascorbic acid is administered, at times, with mercurial diuretics since in experimental animals it protects against cardiac arrest, which is one of the toxic effects of these agents. It has been assumed that ascorbic acid acts as a reducing agent in lowering the toxicity of the mercurial. Recent studies indicate that sizable doses of ascorbic acid may interfere with the action of mercury as a diuretic, blocking the depression of maximum tubular excretion affected by mercurhydrin.⁶³ Further investigation of ascorbic acid and the metabolism of drugs appears to be warranted.

In recent years a considerable body of evidence on the effects of ascorbic acid on resistance to infectious organisms has been accumulated through investigations in experimental animals.⁵⁹ Both positive and negative effects have been reported. In those cases in which ascorbic acid has been shown to promote disease resistance, its role has remained obscure. There have been no good studies of ascorbic acid nutriture in human subjects with infectious diseases under controlled situations using modern methods. Vitamin C requirements during infection remain unknown, and the value of therapy of infections with ascorbic acid has not been determined. A number of therapeutic trials with vitamin C have been conducted and many extravagant claims have been made. In one careful study vitamin C was found to have no beneficial effect on the incidence and cure of the common cold.⁶⁰

Vitamin C has been recommended in the treatment of rheumatoid arthritis and osteoarthritis in combination with hesperidin, without convincing evidence of benefit.⁶¹ Ascorbic acid has been reported to be of value in the treatment of burns, reducing the time interval necessary for skin grafting.⁶² Therapy with ascorbic acid has been recommended in the management of hypermenorrhea, nausea and vomiting of pregnancy, miscellaneous types of cardiovascular and cerebrovascular diseases, and even in atherosclerosis. It has also been recommended in the treatment of habitual abortion and in the therapy of acute

ulgaris. The therapeutic value of vitamin C in these diverse pathologic states has not been established.

Summary

The minimal quantity of ascorbic acid that will prevent scurvy is approximately 10 mg. daily in infants and slightly less than this in adults. Considerable evidence indicates that the desirable intake for the maintenance of health is much larger than this. In guinea pigs more ascorbic acid is needed for growth and for certain metabolic functions than for prevention of scurvy. In infants and cow's milk, a daily supplement of 20 mg. of vitamin C is required to bring plasma ascorbic acid concentration up to that observed in breast-fed infants. Several investigations indicate that a dietary supply of 30 mg. of vitamin C is necessary for the maintenance of healthy gums. In adults a daily intake of less than 40 mg. of ascorbic acid is associated with rapid depletion of tissue stores; an intake of 60 to 100 mg. is needed for saturation or near saturation of the tissues. From a teleological standpoint, saturation may be the desirable state in man, since in all animals that synthesize vitamin C the tissues are saturated with the vitamin.

Vitamin C requirement is increased during growth and in pregnancy and lactation. Considerable evidence suggests that requirement is increased by trauma, infections, and other stress situations.

Determination of optimal ascorbic acid intake must await further knowledge of the function of this vitamin and the development of tests indicative of early functional impairment. In the light of present knowledge, provision of sufficient ascorbic acid to maintain near saturation of the tissues would appear to be desirable.

The primary use of ascorbic acid in clinical medicine is in the prevention and treatment of scurvy. Adequate supplies should be provided during pregnancy and lactation to fulfill increased needs. Ascorbic acid is important in the prevention of megaloblastic anemia of infancy and may be useful in certain other types of macrocytic anemia. Ascorbic acid in large quantities increases the absorption of iron from the intestinal tract. Ascorbic acid is necessary for the normal healing process and is often administered in larger than normal amounts following trauma and in infections. The therapeutic value of large doses of ascorbic acid in these situations has not been determined with certainty. Therapy with ascorbic acid has been advocated in many diverse pathologic states without definite evidence of benefit.

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ASCORBIC ACID ECONOMY IN SURGICAL PATIENTS*

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Although ascorbic acid is known to be necessary for wound healing^{1,2} and heretofore of extreme importance to the surgical patient, its more or less precise requirements remain controversial, having been subject to a variety of estimates.³⁻⁵ More recently the surgical requirements for vitamin C have demanded even further revaluation in view of the suggestion that stress accentuates the need for the vitamin and that the seriously injured human "behaves physiologically like the scorbutic."⁶ True, ascorbic acid is readily available, inexpensive, and nontoxic,⁷ and probably all conceivable demands can be met by the administration of 1 gm. daily. To those interested in a more lucid interpretation of the actual physiological processes involving this vitamin, however, a more exact knowledge of the body requirements imposed by major surgery and/or its complications would seem desirable.

Method

This report concerns the body economy of ascorbic acid in surgical patients, chiefly as reflected by their blood levels of the vitamin and their clinical response. Plasma and buffy coat ascorbic acid were determined as dehydroascorbic acid, using the method of Roe and Kuether,⁸ the buffy coat being separated by the method of Butler and Cushman.⁹ Determinations were made on consecutive days on several pints of freshly stored blood. Thereby the standard deviation and 95 per cent confidence limits for both plasma and buffy coats were obtained. Statistical evaluation showed that the individual plasma determination in our laboratory had a standard deviation of ± 0.09 mg. per cent and a 95 per cent confidence limit of ± 0.19 mg. per cent. For the buffy coat, the standard deviation was ± 1.89 mg. per 100 gm. and the 95 per cent confidence limit ± 3.7 mg. per 100 gm. These latter errors may be much larger when there is difficulty in separating a clear buffy coat.

Average Blood Levels

Over a 10-year period blood ascorbic acid determinations have been performed on surgical patients of all types, exclusive of burns, at the Boston City Hospital. Throughout this period normal people were repeatedly sampled, as were all available clinical scorbutics on the medical wards (TABLE 1).

The average plasma ascorbic acid level in 20 normals was 0.69 mg. per cent, the buffy coat ascorbic acid 15.0 mg. per 100 gm. Among 20 scorbutics tested, these values were 0.08 mg. per cent and 3.6 mg. per 100 gm. respectively. The average levels for 100 consecutive surgical patients were 0.36 mg. per cent and 14.5 mg. per 100 gm. respectively.

As a working hypothesis throughout this report, we shall postulate that

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plasma ascorbic acid below 0.2 mg. per cent and a buffy coat level below 8 mg. per 100 gm. represent serious vitamin C deficiency. These are the levels suggested by the work of Bartlett *et al.*¹

Blood ascorbic acid determinations have now been performed on a total of 75 surgical patients whose average plasma and buffy coat ascorbic acid levels turn out to be a close approximation to the averages of the first 100 sampled

TABLE 1
BLOOD ASCORBIC ACID*

Plasma (mg. %)	Patients	Buffy coat (mg./100 gm.)
0.69	Normal (average of 20 normals)	15
0.36	Surgical patients (average of 100 consecutive patients, Boston City Hospital)	14.5
0.20	Seriously deficient (estimated)	8
0.08 (average of 20 patients)	Clinical scorbutus	3.6 (average of 15 patients)

* Roe-Kuether method.

TABLE 2
BLOOD ASCORBIC ACID AND WOUND DEHISCENCE, GROSS OR CONCEALED
IN ALL SURGICAL PATIENTS*

Total groups sampled	No.	Per cent	Average blood ascorbic acid at time of first sampling	
			Plasma (mg. %)	Buffy (mg./100 gm.)
Surgical patients	875		0.36	13.7
Deficient surgical patients	272		0.11	5.4
Surgical patients subsequently subjected to laparotomy	287		0.37	14.9
1. With sufficient blood ascorbic acid levels	172	60	0.52	18.9
2. With deficient blood ascorbic acid levels	115	40	0.11	5.2
3. "Blood sufficient" patients (C1) subsequently developing dehiscence	3	1.7	0.46	21.1
4. "Blood deficient" patients (C2) subsequently developing dehiscence	16	13.3	0.12	6.9

* Boston City Hospital.

TABLE 2), being 0.36 mg. per cent and 13.7 mg. per 100 gm. respectively. Of these, 272, or 31 per cent, showed deficient blood ascorbic acid levels according to our standards.

Impaired Wound Healing

To assess statistically the rate of impaired wound healing, those patients who had had blood ascorbic acid determinations performed at time of definitive surgery were reviewed (TABLE 2, group C). This group comprised a total of

287 patients of whom 172 had sufficient blood ascorbic acid levels, while 115 had deficient blood levels of the vitamin, according to our standards.

Among the blood ascorbic acid-sufficient group, three patients subsequently developed wound dehiscence: a dehiscence rate of 1.7 per cent. This rate compares favorably with the dehiscence rate of 1.6 per cent reported by Marshall *et al.* among 500 well-nourished patients in a private hospital.¹⁰ Among the 115 blood ascorbic acid-deficient patients, on the other hand, there were 16 wound dehiscences: a rate of 13.9 per cent. These patients are documented in TABLE 3. There was thus an eightfold increase in wound dehiscence rate in the blood ascorbic acid-deficient group as compared to the group showing adequate levels of the vitamin. The increased plasma and buffy coat ascorbic acid averages among the blood-deficient patients subsequently developing dehiscence (TABLE 2, group C4) over the respective averages of the entire group with deficient blood ascorbic acid levels (TABLE 2, group C2) result from ascorbic acid having been given to some patients between the time of their initial analysis (TABLE 2, group C2) and their subsequent dehiscence (TABLE 2, group C4). Data is documented in TABLE 3.

Forty-two other patients were seen and had blood analyses for the first time at the time of wound disruption. There was thus a total of 63 patients suffering wound dehiscence who had blood ascorbic acid determinations performed at the time of this catastrophe. Thirty-six, or 57 per cent of this total, showed serious deficiency of blood ascorbic acid at time of dehiscence. Compared to the total group of 287 surgical patients of which 40 per cent were deficient, this distribution of deficient patients is significantly higher when tested by the χ^2 test. Thus the present results show that a deficient blood ascorbic acid level is frequently associated with wound dehiscence. Since many other factors (such as coughing or straining, infection, or inadequate wound closure) frequently play an important part in the disruption of the surgical wound, it is not possible to say that vitamin C deficiency alone caused a given dehiscence, or vice versa. It can be said, however, that maintenance of the patient's blood ascorbic acid at an adequate level appears to be a potent means of preventing wound dehiscence.

Diagnosis of Concealed Dehiscence

The cardinal sign of concealed dehiscence is serosanguinous discharge from the wound. In suspecting ascorbic acid deficiency as a cause, the dietary history is of greatest importance. On the other hand, the clinical appearance of the wound is not always a reliable guide in making such a diagnosis. An example of this was seen in a patient who displayed the classic signs of ascorbic acid deficiency in his draining wound, with subcutaneous ecchymoses and minute hemorrhages around each suture 8 days after a subtotal gastrectomy (FIGURE 1). However, this patient's blood ascorbic acid level was within normal limits and responded normally to 1-gm. doses of ascorbic acid on 3 consecutive days. Moreover, the wound looked essentially the same a week later despite large doses of the vitamin. A coagulase-positive staphylococcus was cultured from this wound and was the cause of its appearance.

Where the dietary intake of the vitamin has been low, where the serosan-

TABLE 3
SIXTEEN BLOOD ASCORBIC ACID-DEFICIENT PATIENTS UNDER STUDY, SUBJECTED TO
LAPAROTOMY AND SUBSEQUENTLY SUFFERING WOUND DEHISCENCE

Patient, age, hospital No.	Procedure	Predehiscence blood		Postop. vitamin C		Type of dehiscence postop.	Blood ascorbic acid at time of dehiscence		Outcome
		Plasma (mg. %)	Buffy (mg./100 gm.)	Amount (mg./day)	Commenced (postop. day)		Plasma (mg. %)	Buffy (mg./100 gm.)	
H.C., 73, 1371831	Exptl. lap. for chr. volvulus	0.06	7.8	100	7	Gross	0.06	0.0	Recovered
J.L., 84, 1364789	Gastrectomy	0.19		100	1*	Gross	0.19	0.0	Died
D.G., 64, 1368943	Rupt. append.	0.15		0	—	Gross	0.15		Recovered
S.I., 62, 1447408	Exptl. lap. with strang. hernia	0.14	7.0	500	4	Gross	0.00	0.0	Died
H.W., 64, 1504579	Perforated ulcer closure	0.08	8.0	100	1*	Gross	0.09	5.6	Recovered; large hernia
S.M., 61, 1690632	Cholecystectomy	0.04	2.9	125	9	Gross	0.47	7.3	Recovered
J.D., 73, 1379269	Append. and hernia	0.11	0.0	0	—	Gross	0.84	18.4	Recovered
H.H., 70, 1374813	Cholecystectomy	0.17	12.9	0	—	Gross	0.15	17.5	Died
L.McL., 65, 1477269	Perforated ulcer closure	0.11	7.8	100	1*	Gross	0.19	4.5	Recovered
E.M., 77, 1490127	Perforated ulcer closure	0.12	8.4	100	14	Gross			Recovered; large hernia
M.K., 79, 1505461	Exptl. lap. cholecystectomy	0.14		0	—	Gross			Died
J.F., 65, 1424179	Subtotal gastrectomy	0.14	14.5	0	—	Concealed	0.09	4.3	Recovered; large hernia
G.R., 39, 1460230	Subtotal gastrectomy	0.19	2.6	75	1*	Concealed	0.19	13.2	Recovered
C.C., 58, 1402211	Subtotal gastrectomy	0.12	8.3	0	—	Concealed			Recovered
P.K., 53, 1386212	Total gastrectomy	0.12	3.1	100	3	Concealed	0.20	4.4	Recovered; large hernia
H.L., 76, 1489768	Exptl. lap. cancer of stomach	0.12		0	—	Concealed	0.09	6.5	Recovered

* Beginning immediately postop.

guinous discharge from the wound proves to be sterile on culture, and where a large incisional hernia later develops, ascorbic acid deficiency is a more likely cause of the partial or concealed wound dehiscence. An example was a 54-year-old male who was initially very deficient in ascorbic acid, had a total gastrectomy, and was carried through his entire convalescence on only 1000 mg. ascorbic acid per day. His entire postoperative course has been previously reported in detail.¹¹ On his 14th postoperative day this patient developed serosanguinous drainage from his wound that proved to be sterile on culture. By his 6th postoperative week he had a large incisional hernia (FIGURE 2).

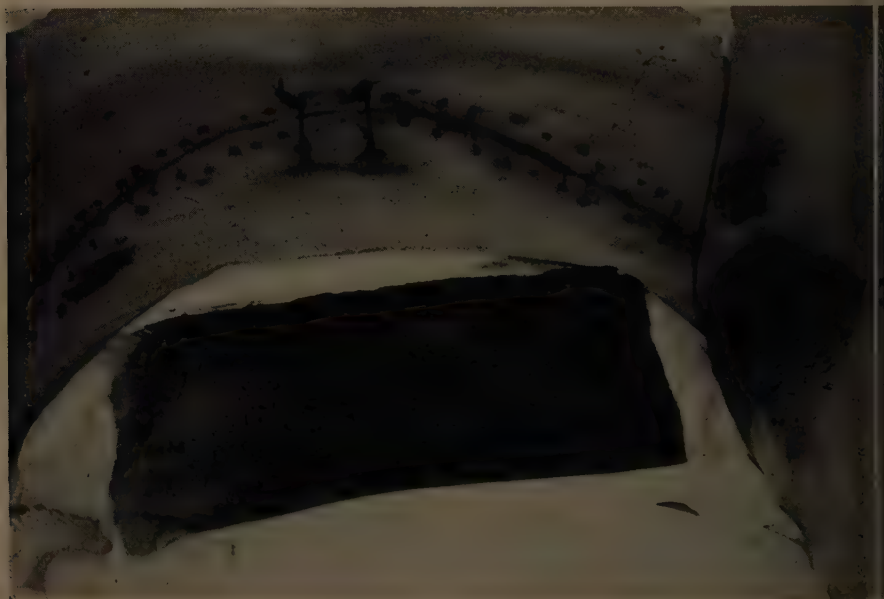


FIGURE 1. Concealed dehiscence 8 days after subtotal gastrectomy. Serosanguinous discharge, subcutaneous ecchymoses, and minute hemorrhages around each suture.

Wound Biopsy

Attempts to prove ascorbic acid deficiency by biopsy of the dehiscd wound of surgical patients have generally been unsuccessful, since before gross separation of the wound occurs, an intense inflammatory response is set up in the wound edges that obscures any microscopic study of it. Biopsy of one disrupted wound in this series was sufficiently free of inflammatory response to show on microscopic examination young fibroblasts laying down some collagen (FIGURE 3). This patient was of particular interest since his plasma ascorbic acid at time of wound dehiscence was zero, whereas the wound tissue itself was found to contain 1.3 mg. of ascorbic acid per 100 gm. of tissue (TABLE 4). Unfortunately, a satisfactory buffy coat could not be obtained from this patient's blood sample.

Biopsies of open, clean granulations are more often satisfactory. Thus biopsy of the clean open granulations of an amputation stump of an ascorbic acid-deficient patient with obliterative peripheral vascular disease showed on microscopic examination a typical picture of scorbutus (FIGURE 4). This patient had a plasma ascorbic acid level of 0.12 mg. per cent, a buffy coat level of 6.0 mg. per 100 gm.



FIGURE 2. Large incision hernia 6 weeks after total gastrectomy. Patient maintained 100 mg. of ascorbic acid daily. Drainage from sinuses sterile on culture.

Tissue Analysis of Disrupted and Granulating Wounds

The tissues of dehiscenced wounds were analyzed for ascorbic acid content in several patients. Tissue determinations were also performed at time of the primary operative procedure (TABLE 4). Among patients with a normal economy of the vitamin, the ascorbic acid content of the dehiscenced or granulating wound was found to be considerably higher than that of the normal tissues of the blood, ranging from 5 to 21 mg. per 100 gm. as compared to values of 1 to 2 mg. per 100 gm. for normal rectus fascia. These findings coincide with those found by Abt and von Schuching (elsewhere in this monograph) and Schauble *et al.*¹² in guinea pigs. However, where severe infection is present in the wound or where the patient is markedly deficient in vitamin C, the ascorbic acid content of the wound will be much lower. Thus patient J.L., who had relatively adequate blood levels of the vitamin, showed only 0.05

mg. of ascorbic acid per 100 gm. of tissue in his severely infected and disrupted wound after a Miles resection; patient M.B., who had deficient blood levels of the vitamin, showed only 0.3 mg. of ascorbic acid per 100 gm. in his wound at time of evisceration subsequent to gastrectomy (TABLE 4, patients 9 and 10). The same phenomenon of increased tissue concentration of ascorbic acid was seen not only in the dehiscence wound but also in granulating surfaces such as



FIGURE 3. Young fibroblasts laying down some collagen in disrupted wound edges of patient having no ascorbic acid or plasma.

chronic gastric ulcer (TABLE 4, patient 5) and a granulating thoracotomy wound (TABLE 4, patient 6).

Maintenance Studies

Maintenance studies were performed on 26 patients, who in general were supplied with the minimal amount of ascorbic acid compatible with surgical recovery and adequate blood ascorbic acid levels. An exception to this was patient with ruptured appendix, peritonitis, and intestinal obstruction (FIG-

TABLE 4
BLOOD AND TISSUE ASCORBIC ACID IN PATIENTS WITH
HEALING WOUNDS AND GRANULATIONS

Patient*	Age	Blood ascorbic acid (mg. %)		Tissue ascorbic acid (mg./100 gm.)		
		Plasma	Buffy	Fascia	Muscle	Granulations
1. D.F. Postop. (8)	62	0.32	23	1.9	4.0	
2. J.M.†	11	0.64	16.0			21.0
3. G.R.	55	0.70	11.5			8.0
4. F.M.	55	0.34	13.0			18.0
5. H.J.‡	65	0.00				1.3
6. E.M.†	65	0.51	28.6	2.0	4.0	14.0
7. F.B.†	41	0.35	14.3	1.3	1.8	8.4
8. S.W.	53	0.62	14.0			5.0
9. M.B.†	43	0.99	19.0	1.4	3.0	
10. J.L.†	58	0.11	4.0	0.3		
		0.31	11.8	0.05		

* Diagnosis and procedures:

1. Cancer of the cecum; exploratory laparotomy, evisceration closure.
2. Evisceration closure; postappendectomy.
3. Concealed evisceration; postgastrectomy.
4. Evisceration closure; postduodenostomy.
5. Gastric ulcer; gastrectomy.
6. Pneumonectomy; postthoracotomy.
7. Evisceration closure; postgastrectomy.
8. Evisceration closure; postgastrectomy.
9. Evisceration; postgastrectomy.
10. Evisceration; post Miles resection.

† Previously reported.¹¹

‡ Previously reported.¹⁸

E 5). He was on constant gastric suction for a prolonged period so that control of his oral intake of the vitamin was no problem. Directly after second operation for release of an obstruction, he was started on 100 mg. of ascorbic acid each day intravenously. On this maintenance dose his deficient blood ascorbic acid levels showed no improvement. After 8 days of this regimen he was started on orange juice by mouth; his wound infection cleared, and he rapidly recovered.

A 43-year-old woman with a poor dietary intake and low blood level of the vitamin was subjected to one of the most extensive of surgical procedures, a radical vulvectomy and bilateral groin dissection for carcinoma of the vulva. She withstood the surgery well. Postoperatively, because of her depleted



FIGURE 4. Young fibroblasts with no intercellular substance in granulation of patient with deficient blood levels of ascorbic acid (reproduced by permission of *New England Journal of Medicine*).

ate, she was placed on 600 mg. of ascorbic acid intravenously each day for 6 days (FIGURE 6). During this time her plasma and buffy-coat ascorbic acid levels rose markedly in spite of a severe urinary infection. After 6 days the ascorbic acid supplement was reduced to 200 mg. daily, a dose that was maintained throughout the remainder of her hospital course. In the first two weeks following surgery the skin flaps of both groin dissections sloughed—a complication almost standard following this type of operation. Such a complication sets up a tremendous area of inflammatory response. Nevertheless, her wounds at the end of three weeks were filled with healthy looking granulations (FIGURE

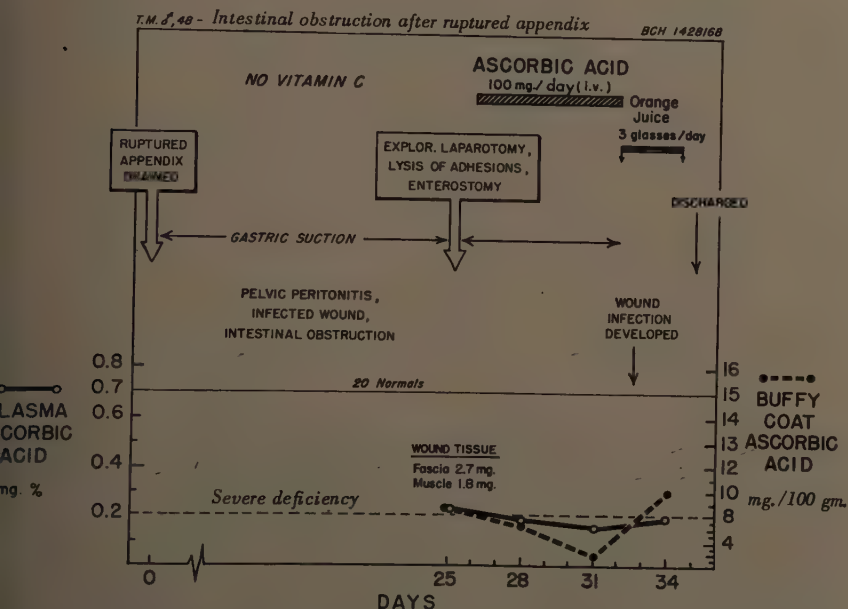


FIGURE 5. Maintenance studies: patient with ruptured appendix on constant gastric suction for intestinal obstruction. Maintained on 100 mg. vitamin C per day; diet prior to hospitalization fair in fruit and vegetables.

On this patient's 26th postoperative day, an extensive skin grafting operation was performed to cover the raw surfaces of her wounds. With this operative procedure, there was a tremendous fall in her buffy-coat ascorbic acid with only a slight fall in her plasma level of the vitamin. Thereafter there was a rapid rise in her buffy-coat ascorbic acid until infection and slough of the skin flaps occurred on her 34th day. At this time, there was again a severe, more persistent fall in her buffy-coat vitamin level without associated fall in her plasma ascorbic acid. This pattern of high plasma with low buffy-coat ascorbic acid levels suggests that when the patient is receiving daily parenteral supplements of ascorbic acid, the plasma level of the vitamin may be much higher than a true reflection of the body economy would indicate.

Another patient, having a wide and deep excision of a large area of the back

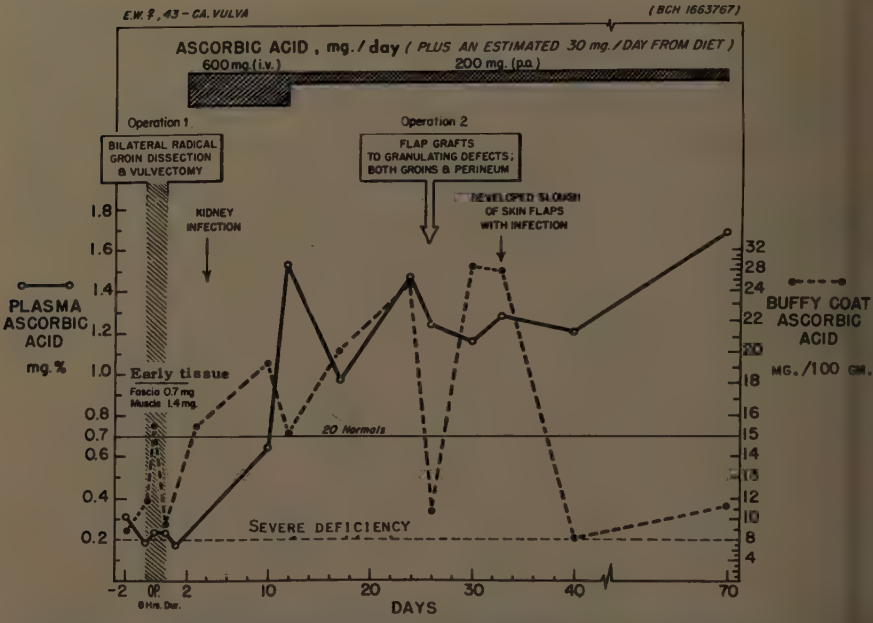


FIGURE 6. Maintenance studies: deficient patient undergoing severe operative procedure; diet prior to surgery very deficient in fruit and fresh vegetables (see FIGURE 7).



FIGURE 7. Extensive slough of wound edges with healthy granulation tissue in wound defects 3 weeks after radical vulvectomy with bilateral groin dissection. Patient E.W., FIGURE 6.

and radical groin dissection on one side for malignant melanoma of the back, as maintained throughout his entire course on a supplement of 200 mg. of ascorbic acid per day (FIGURE 8). He also received an estimated 50 mg. of the vitamin daily in his diet. On this intake his blood plasma and buffy-coat levels rose to well-above-normal values during convalescence from his first surgery, which was uncomplicated by infection or skin slough. When he was subjected to a prophylactic axillary dissection 18 days later, there was a marked fall in both plasma and buffy-coat levels, with subsequent rapid recovery again. In these studies, the highest daily supplement of ascorbic acid found necessary to maintain borderline deficient blood levels of the vitamin was 300 mg. in the

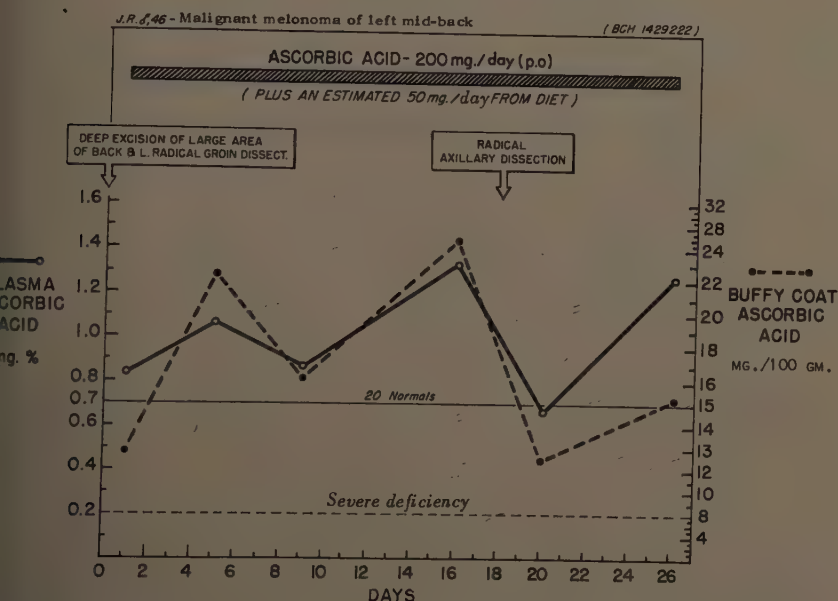


FIGURE 8. Maintenance studies: radical groin dissection, extensive excision of skin of back and subsequent radical axillary dissection; diet prior to hospitalization good in fruit and vegetables.

of a patient with severe and ultimately fatal ulcerative colitis (FIGURE 9). This 33-year-old man had been discharged from the hospital with an ileostomy and with relatively normal blood ascorbic acid levels, on a maintenance dose of 100 mg. of ascorbic acid daily. After approximately one year, during which time he was lost to follow-up, he re-entered the hospital in poor condition with multiple draining perineal fistulae and profuse discharge from his ileostomy. At time of re-entry, this patient's plasma ascorbic acid was 0.10 mg. per cent, and buffy coat 6.6 mg./100 gm. He was started on 100 mg. of the vitamin daily by intramuscularly, being unable to take anything by mouth. On this regimen, his buffy coat fell, so the dosage was increased to 200 mg. of ascorbic acid daily. His blood levels continued to fall, reaching 0.06 mg. per cent for plasma and 0.0 mg. per cent for the buffy coat. At this point his dose of vitamin C was increased to 300 mg. daily. This was followed by only a modest

rise in his plasma, and maintenance of his buffy coat was at a borderline deficient level. It is likely that 400 mg. of ascorbic acid daily would have been required to maintain this patient's blood levels of the vitamin at adequate values during the terminal phase of his severe illness. Here again, the daily parenteral vitamin supplement appears to have raised the plasma vitamin C level beyond a level reflecting the true state of the tissue economy, as indicated by the buffy-coat ascorbic acid. This patient's maintenance study was complicated by the administration of cortisone for 6 days, but it should be noted that during the last 3 days of cortisone therapy his blood levels of the vitamin rose.

From these and similar studies the impression is gained that the amount

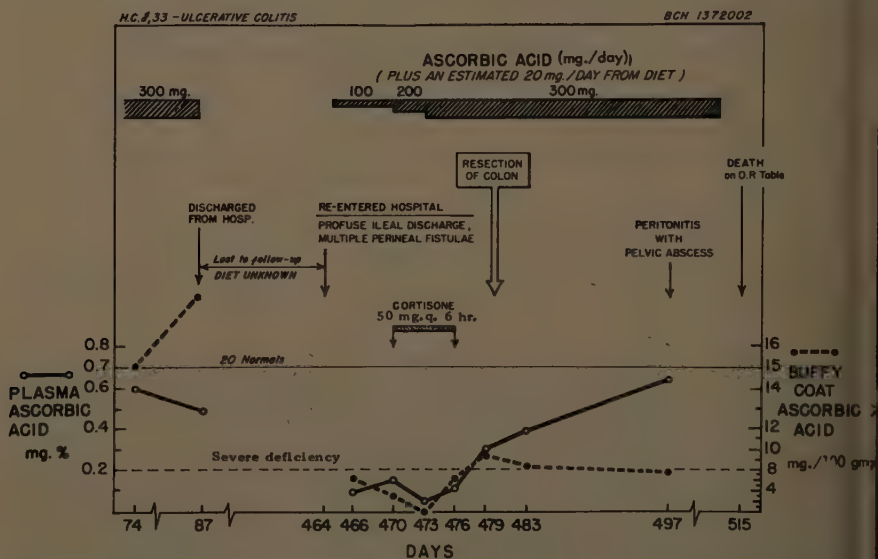


FIGURE 9. Maintenance studies: severe fatal ulcerative colitis requiring ileostomy and staged colectomy.

of ascorbic acid required varies with the degree of inflammatory response and that for surgical patients (exclusive of burns) this seldom exceeds 400 mg. daily.

Surgical Complications Other Than Wound Dehiscence

Other than wound dehiscence, what deleterious effects were possibly the result of surgery upon the ascorbic acid-deficient patients in this study? Among 272 patients of all types with deficient blood ascorbic acid levels subjected to surgery, the following complications possibly related to ascorbic acid deficiency were found (TABLE 5): two patients suffered generalized oozing of blood from their wounds, one with fatal outcome. The fatal case (J.W.) had been given 1300 mg. of ascorbic acid in the 24 hours prior to surgery, yet his tissues at operation showed an extremely low level of the vitamin. Although this man had cirrhosis of the liver, his prothrombin time, as well as his bleeding and clotting times, were normal.

One patient developed complete paraplegia below the level where his cord was tapped by a spinal needle for continuous spinal anesthesia. It is quite possible that a hematoma formed at this site, which later set up a paralyzing adhesive arachnoiditis. This was the neurological diagnosis. Lastly, a patient with fractures of both bones of his lower leg and severe urinary infection was given daily for 2 months only a 100 mg. supplement of ascorbic acid. His blood levels of the vitamin were extremely deficient for the first 3 weeks but improved thereafter. His fractures showed no evidence of healing by X ray

TABLE 5

SURGICAL CATASTROPHES OTHER THAN WOUND DEHISCENCE POSSIBLY
RELATED TO ASCORBIC ACID DEFICIENCY

Patient Op. No.	Age Diet	Operative procedure	Blood ascorbic acid		Course
			Plasma	Buffy	
7.* 1905	62 Poor	Repair of large ventral hernia	0.07	2.8	Died 24 hours after surgery of shock with generalized oozing into wound
M. 2353	51 Poor	Bilateral vein ligation with stripping	0.14	6.4	Generalized slow oozing into wounds for 3 days, requiring gel-foam packs
945	54 Poor	Gastrectomy under continuous spinal	0.11	5.0	Developed adhesive arachnoiditis and complete paraplegia, possibly secondary to hematoma of cord from spinal
919	65 Poor 50 mg. C/DA to day (60)	Open reduction of fractures, both bones, lower leg, with bone graft	0.06 (1) 0.12 (13) 0.43 (56) 1.17 (148)	0.0 2.0 11.5 17.1	Persistent urinary infection. No healing of fracture seen for 1½ years. Commencing day (60) vitamin C increased to 200 mg./day

Received 1300 mg. vitamin C I.V. 24 hours prior to these determinations. Wound healed at op. 0.1 mg./100 gm.

1½ years. In this case the question arises whether a poor callus was formed during the first month after this patient's injury, which later retarded bone formation.¹³

Immediate Effects of Surgery

The immediate effects of surgery upon plasma and buffy-coat ascorbic acid levels were studied in approximately 150 cases. The over-all average drop in plasma ascorbic acid was 17 per cent; in buffy-coat ascorbic acid it was 20 per cent. In a few cases there was a slight rise. Although the pattern was not means uniform, the general impression gained was that in general the patients with the higher blood levels of the vitamin showed the sharpest drop,

those with the lower levels a more gradual drop in blood ascorbic acid during surgery (FIGURE 10). No correlation between the degree of fall, the type and duration of operation, or the type of anesthesia could be found. In a few de-

BUFFY-LAYER VITAMIN C LEVELS - RESPONSE TO OPERATION

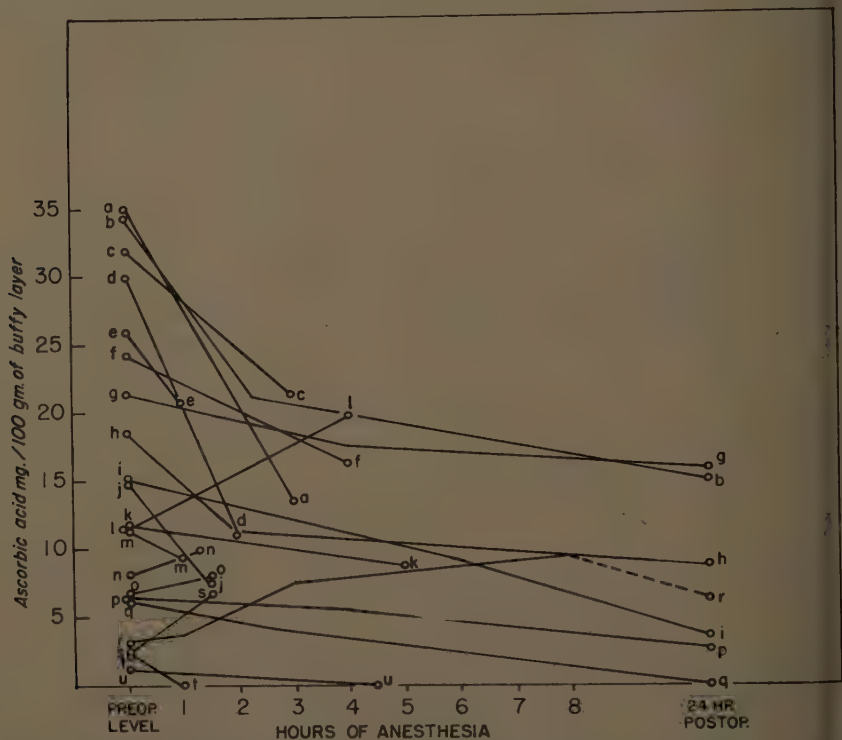


FIGURE 10. Buffy-coat ascorbic acid values plotted against hours of operation up to 6 and 24 hours in 24 patients undergoing major surgery. Key: a. ulc. colitis: ileostomy, R. colectomy (S, P); b. hemithyroid (P, C, NO); c. choledochotomy (S, NO, C); d. mitral valvuloplasty (P, C, GOE); e. prostate (S); f. partial gastrectomy (P, NO); g. partial gastrectomy (S, P, NO); h. colostomy (S, P); i. subtotal gastrectomy and cholecystectomy (EE); j. exploratory lap. (GOE); k. partial gastrectomy (P, C, GOE); l. partial gastrectomy (C, P, EE); m. gall bladder (S); n. sympathectomy (S); o. colostomy (S); p. partial gastrectomy (S, P, EE); q. excise cervical mass (P, C, NO); r. total gastrectomy and splenectomy (P, C, EE); s. hernia (S); t. exploratory lap. (S); u. partial gastrectomy (S, P, NO).

The parenthetical letters indicate type of anesthesia: S, spinal; NO, nitrous oxide; P, pentothol; GOE, gas, oxygen, ether; C, cyclopropane; EE, ether ethylene.

ficient patients the blood ascorbic acid fell to zero without immediate noticeable deleterious effect.

In 10 patients the rectus fascia and muscle ascorbic acid was determined at the beginning and again at the end of the long surgical procedures. No appreciable change in wound tissue ascorbic acid during surgery could be found.

Among 9 patients given 1000 mg. of ascorbic acid intravenously during surgery, no appreciable change could be found in the wound ascorbic acid a

of surgery except in one patient, G.G., undergoing prostatectomy, whose blood was flooded with vitamin C-enriched urine during the operative procedure (TABLE 6). It will be noted that among this group of patients, in all but one the plasma ascorbic acid jumped to high levels whereas the buffy-coat ascorbic acid rose only moderately. These findings, together with those of patient J.W. (TABLE 5), suggest that to raise the tissue ascorbic acid level for surgery, the vitamin should be given for some time beforehand. In this group of patients the preoperative and postoperative urines were also analyzed for ascorbic acid content using the method of Evelyn *et al.*¹⁴ There

TABLE 6

BLOOD, WOUND, AND URINARY ASCORBIC ACID AT BEGINNING AND END OF SURGERY IN NINE PATIENTS RECEIVING 1000 MG. ASCORBIC ACID DURING OPERATION

Patient*	Ascorbic acid (mg. %)				Tissues (mg./100 gm.)				Urinary ascorbic acid (mg./l.)	
	Plasma		Buffy		Fascia		Muscle			
	Preop.	Post-op.	Preop.	Post-op.	Preop.	Post-op.	Preop.	Post-op.	Preop.	Postop.
K.	0.23	2.49	7.0	7.0	0.8	1.4	2.3	2.6	45	720 (330 cc.)
K.	0.00	1.80	2.5	8.1	0.9		1.0		135 (45 cc.)	5920 (25 cc.)
C.	0.12	1.38	4.0	7.0	1.0	1.0	2.0	1.7	45 (125 cc.)	2000 (25 cc.)
R.	0.11	1.44	1.1	1.5	1.4	1.5	0.6		30 (210 cc.)	2445
G.	0.35	2.53	6.0	10.0	0.9	4.0	2.0	5.0	45 (86 cc.)	450 (200 cc.)
R.	0.00	1.80	2.5	8.1	1.0	1.0	2.0	1.7		60 (118 cc.)
L.	0.07	0.39	0.0	1.0	0.0	0.1	0.0	0.2		840 (22 cc.)
S.	1.03	4.89	13.0	19.0	2.0	0.6	3.0	1.0		2320
M.	0.39	2.49	4.0	7.0		1.0	0.0			1695 (400 cc.)

Diagnosis and procedures:

1. Cancer of sigmoid; sigmoid resection.
2. Chronic thrombophlebitis; sympathectomy.
3. Gangrene foot; mid thigh amputation.
4. Cancer of stomach; gastroenterostomy.
5. Obstructing prostate; prostatectomy.
6. Diverticulitis; sigmoid resection.
7. Diverticulitis; colostomy.
8. Gastric polyp; polyp resection.
9. Cancer of neck; radical neck.

a very large increase in urinary ascorbic acid values after surgery as compared to the preoperative values. However, the tremendous variation in amounts of the vitamin excreted by the deficient patients in this group gives rise to the question of whether there are other variable factors besides deficiency influence the excretion of the vitamin.

Effect of ACTH and Cortisone

What effects have stress and/or surgery per se (apart from drugs¹⁵ or any inflammatory response) upon the body economy of ascorbic acid? Influence of large amounts of steroids upon the blood ascorbic acid levels in free medical patients on a carefully measured diet was documented, with the following results.

One patient (R.C., FIGURE 11) whose intake of ascorbic acid had been high was under treatment for sickle cell anemia and aseptic necrosis of his hip joint. While he received 200 mg. of cortisone and approximately 100 mg. of ascorbic acid in his diet daily, there was a slow fall of his high buffy-coat ascorbic acid level with a slow rise of his plasma levels over a period of 37 days. Another patient (P.M., FIGURE 12) received cortisone intermittently over a long period of time for rheumatoid arthritis. He showed a considerable drop in his high buffy-coat ascorbic acid level each time cortisone therapy was initiated but

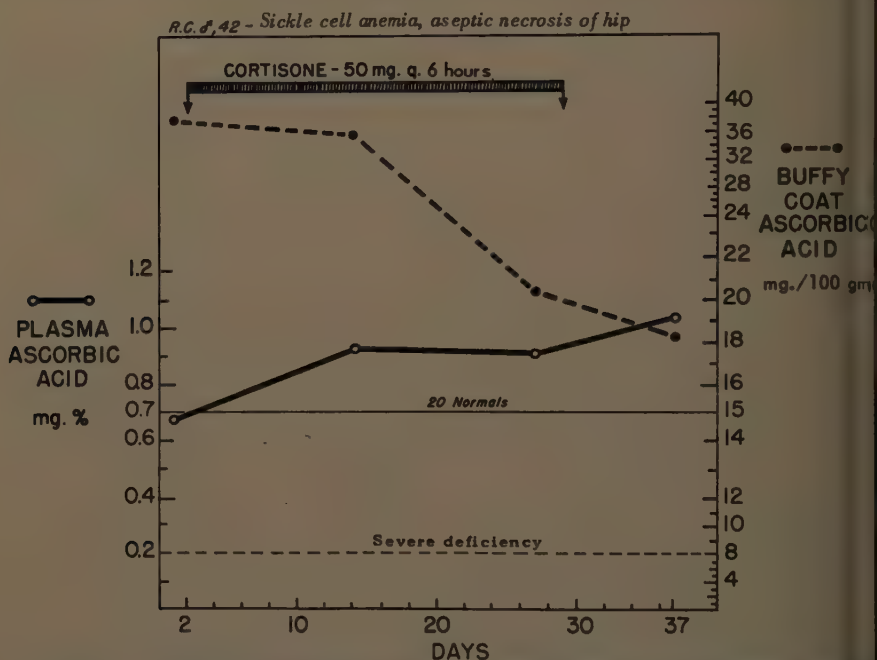


FIGURE 11. Effect of cortisone on blood ascorbic acid levels in a medical patient receiving an estimated 150 mg. per day vitamin C in diet. Courtesy Edward Kass, Thorndike Memorial Laboratory, Boston City Hospital, Boston, Mass.

without much change in his plasma levels of the vitamin. Each time the cortisone was omitted, the buffy-coat ascorbic acid rose slowly again.

On the other hand, another patient with arthritis (J.S., FIGURE 13), whose intake of ascorbic acid had been poor prior to hospitalization and whose blood levels of the vitamin were relatively low, showed relatively little change in blood ascorbic acid during ACTH therapy until he suddenly developed a severe epididymo-orchitis.

From these and similar studies the impression is gained that although the fluctuation of blood ascorbic acid levels associated with excess circulating steroids is variable, there is more likely to be a fall in high blood levels of the vitamin and less likelihood of much change in low blood levels of ascorbic acid with increased body ACTH or cortisone. This pattern is also seen following

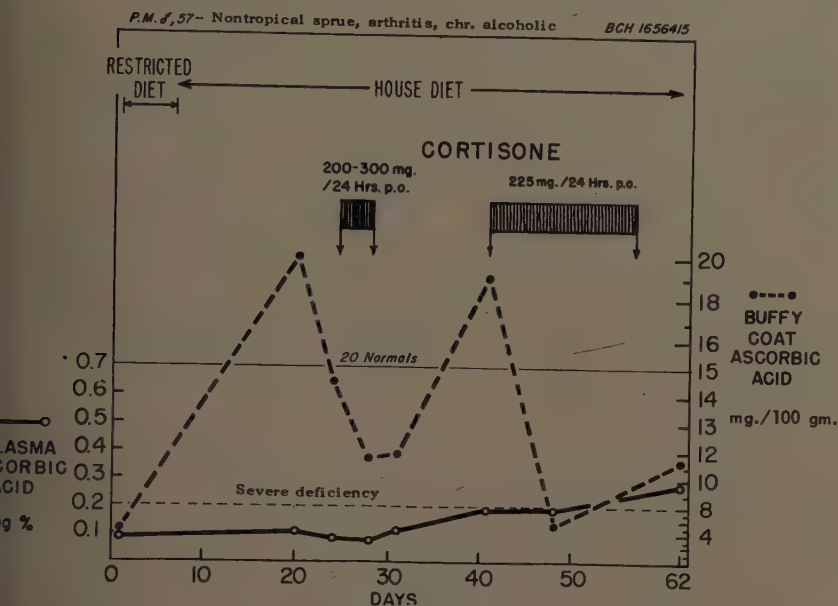


FIGURE 12. Effect of cortisone on blood ascorbic acid levels in a medical patient receiving estimated 50 to 150 mg. per day vitamin C in diet. Courtesy Charles Davidson, Thorndike Memorial Laboratory, Boston City Hospital, Boston, Mass.

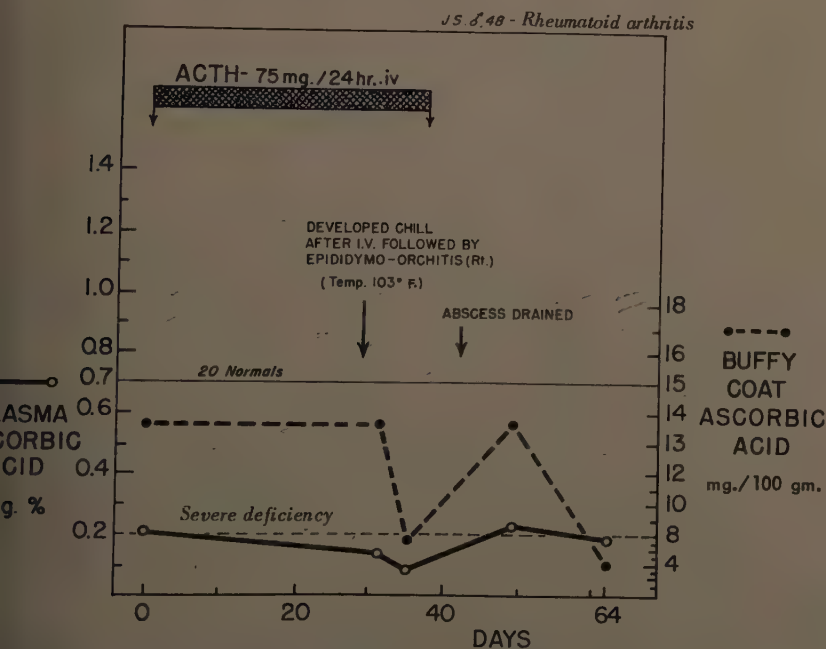


FIGURE 13. Effect of ACTH on blood ascorbic acid levels in a medical patient receiving estimated 100 to 150 mg. per day vitamin C in diet. Courtesy Edward Kass, Thorndike Memorial Laboratory, Boston City Hospital, Boston, Mass.

surgery. It suggests (1) that increased ACTH and/or cortisone cause predominantly a change in body distribution rather than "burning" of ascorbic acid, or (2) that the increased utilization of ascorbic acid by excess steroids is somehow modified or slowed when the body supply of the vitamin becomes low.

Oral Tyrosine Load

In an effort to detect functional impairment of metabolism resulting from ascorbic acid deficiency, oral test loads of 15 to 30 gm. of L-tyrosine have been given to patients, normals and scorbutics with subsequent measurement of 72-hours "tyrosyl" excretion. The urinary "tyrosyl" was measured by the method of Medes.¹⁶ Care must be taken that the patient does not receive aspirin since the phenol rings thereby produced in the urine will give incorrectly

TABLE 7

EFFECT OF 15 TO 30 GM. ORAL LOAD OF L-TYROSINE ON URINARY "TYROSYL" EXCRETION

Type of patient (number tested)	Blood ascorbic acid (mg. % or 100 gm.)		Pretest "tyrosyl" (1 day \times 3)	Range of values	Tyrosine dose	"Tyrosyl" excretion 72 hr. after dose	Range of values
	Plasma	Buffy					
1. Ambulatory normals (7)	0.83	30.0	850	399-1230	30	3086	2332-3590
2. Resting saturated surgical patients (7)	0.45	18.0	715	420-918	30	1957	1510-2460
3. Clinical scorbutics							
a. (2)	0.05		1221		15	5419	5419-5550
b. (5)	0.07	4.0	906	795-1164	30	5726	4387-6990
4. Blood ascorbic acid-deficient nonscorbutics							
a. (5)	0.13	6.7	1769	438-3930	30	4940	4372-6230
b. (2)	0.06	6.8	1651	1035-2267	30	2350	679-4020
c. (1)	0.11	2.1	591		15	3164	

high values of "tyrosyl." As first noted by Rogers and Gardner¹⁷ among clinical scorbutics so tested, we have invariably found a marked increase in the 72-hour excretion of urinary "tyrosyl," ranging in amounts from roughly 4000 to 7000 mg. (TABLE 7).

In a preliminary study we also reported increased tyrosyl excretion after a 30-gm. L-tyrosine load among a small number of surgical patients having deficient blood ascorbic acid levels but no evidence of clinical scurvy (TABLE 7, type 4a). No such increased tyrosyluria had been found by Steele *et al.*,¹⁸ but none of their patients had really deficient blood ascorbic acid levels according to our standards. Unfortunately, as the number of blood ascorbic acid deficient, nonscorbutic patients has increased, cases showing no great increase in tyrosyluria after a tyrosine load have appeared (TABLE 7, type 4b). The possibility that this is due to variations in diet is under investigation.

After major surgery, either with or without an oral tyrosine load, the urinary "tyrosyl" excretion has been found to be even more variable, but in general it is considerably less than after an oral tyrosine load without surgery. The

well illustrated in the instance of a patient who had a bilateral lumbar sym-
 thectomy in two stages, with an oral load of tyrosine before each; he showed
 s tyrosyluria after each surgical procedure than after another oral load of
 rosine without surgery 8 days later (TABLE 8, cases 4A, B, and C). It is ap-
 parent that further investigation is needed along these lines.

At present an effort is being made to correlate increased "tyrosyl" with
 reased 17-ketosteroid excretion after surgery. Thus far, one patient, who
 s subjected to a simple herniorrhaphy without oral tyrosine loading, showed

TABLE 8

EFFECT OF SURGERY UPON BLOOD ASCORBIC ACID, URINARY "TYROSYL," AND
 17-KETOSTEROID WITH AND WITHOUT AN ORAL TEST LOAD L-TYROSINE

Ascorbic acid economy and tyrosine load	Blood vitamin C				Urinary 17-keto		"Tyrosyl" excretion	
	Plasma (mg. %)		Buffy (mg./100 gm.)		Preop.	Postop.	Preop. (1 day × 3)	Postop. (72 hours)
	Preop.	Postop.	Preop.	Postop.				
<i>Saturated:</i> average of 18 pt. each given 30 gm. L-tyro- sine before surgery	0.40		19.4				553	2666
<i>Borderline depleted:</i> average of 5 pt. each given 30 gm. L- tyrosine before surgery	0.15		10.8				248	4049
<i>Individual cases reflecting variation of results</i>								
<i>Depleted:</i> A. One case with 15 gm. L- tyrosine before surgery	0.0		6.4		6.0	9.8	136	1025
<i>Low normal:</i> B. First surgery + 15 gm. L-tyrosine	0.02	0.07	14.7	11.1	6.2	11.8	945	1907
C. Second surgery + 30 gm. L-tyrosine	0.19	0.16	24.4	17.0	7.7	3.5	786	1943
D. Later without surgery + 30 gm. L-tyrosine	0.16		17.0					2830
<i>Low normal:</i> 1 case surgery but no L-tyrosine	0.24	0.0	9.7	0.0	3.3	29.7	1947	4356

marked drop in blood ascorbic acid, a marked rise in urinary 17-ketosteroids,
 a markedly increased tyrosyl excretion after his surgery (TABLE 8, 5).
 In a response would be consistent with the concept that the seriously injured
 stressed person behaves physiologically like a scorbutic. However, no
 titution of the unusual reaction seen in this one patient has so far been ob-
 ed in any other patient studied.

Conclusions

Plasma and buffy-coat ascorbic acid levels are reasonably good indices of
 body economy of the vitamin. As a working hypothesis, a plasma ascorbic

acid content below 0.2 mg. per unit and a buffy-coat level below 8 mg./100 gm. is taken to represent serious vitamin C deficiency.

Among surgical patients subjected to laparotomy, there was an eightfold higher rate of wound dehiscence in those patients with deficient blood levels of the vitamin at primary operation compared to those with adequate levels. It is concluded that a low vitamin C level is associated with wound dehiscence and that maintenance of the patient's blood ascorbic acid at an adequate level preoperatively appears to be a potent means of preventing wound dehiscence.

The majority of surgical patients can be maintained adequately on 100 to 300 mg. of ascorbic acid per day. When such supplements are given, the plasma ascorbic acid level may be high relative to the body status of the vitamin as reflected by the buffy-coat level of the vitamin. Because the buffy-coat and tissue levels rise more slowly, allowance of time for preoperative buildup may be desirable in deficient patients who are candidates for elective surgery.

Following large oral doses, the vitamin level in urine after surgery is a relatively poor index of the body economy of vitamin C; the gross appearance of the wound is also a poor index. A dehiscent or granulating wound will show a considerably higher ascorbic acid content than the primary wound both in normal and in deficient patients, provided severe infection is not present.

Major surgery produces an average decrease of 17 to 20 per cent in plasma and buffy-coat ascorbic acid levels, which is seen more clearly in patients having higher initial levels. A somewhat similar response is observed following administration of ACTH or cortisone.

Although an oral load of 15 to 30 gm. of L-tyrosine produces a marked tyrosyluria in all scorbutics and most patients with deficient blood ascorbic acid levels, such increased tyrosyluria is not seen following surgery in most patients.

Acknowledgments

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METHODS FOR DETECTING AND EVALUATING ASCORBIC ACID DEFICIENCY IN MAN AND ANIMALS

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The assessment of nutritional status with respect to ascorbic acid is more satisfactory than for any of the other nutrients. This is true despite the fact that the cellular biochemical action of ascorbic acid is still poorly understood. The first evaluations of nutritional status were based on clinical findings and the response of patients to dietary therapy. This method is still quite satisfactory for the determination of scurvy, the gross deficiency disease. However, the nutritional spectrum for ascorbic acid, or for any single nutrient, consists of a graded series of possible states with frank deficiency at one end, optimum nutrition beginning at some region in the middle and, possibly, overnutrition at the far end. The transition from optimal to suboptimal nutrition is gradual with few signposts. The difference between optimal nutrition and less than optimal is of practical concern here.

Biochemistry has been of decisive help in the measurement of the nutritional spectrum for ascorbic acid in both animals and human beings. Fortunately, a wealth of biochemical possibilities for the function and the mechanism of action of ascorbic acid has been explored. The extensive studies on the guinea pig have provided much information concerning ascorbic acid metabolism and tissue levels. The results of these studies parallel in many ways the less extensive data on human beings. Experimental results that define possible means for measuring *tissue levels* as an indication of the nutritional status for ascorbic acid follow.

Ascorbic Acid in Experimental Animals

FIGURE 1 presents a series of curves calculated by Lowry¹ from data that Kuether *et al.*² obtained on guinea pigs. The percentage of the maximum level of ascorbic acid in tissues and blood is related to intake. The curves serve to illustrate changes in nutritional status for these animals. In a guinea pig given sufficient ascorbic acid, the tissue concentrations of the vitamin reach ceiling levels that are not exceeded with any reasonable intake. On withdrawal of the ascorbic acid from the diet, the levels fall very low, 3 to 10 per cent of the ceiling. With stabilized diets of differing ascorbic acid content, the tissue concentrations are related to the intake. Except for the curve for blood, the intake tissue concentration curves have approximately the shape of dissociation curves (Giroud, 1938⁶). If a certain intake produces 50 per cent saturation, 2 and 3 times this level would give 75 and 87.5 per cent saturation respectively. The blood level varies with the intake and tissue concentration; however, it is more accurate as an index of status when the tissues are above 50 per cent saturation. The plasma ascorbic acid levels (not shown) at the lower intakes are even less than whole-blood values and are of limited value for assessing status in the range of deficiency.

The ceiling levels of the tissues of guinea pigs from which the curves of FIGURE 1 were calculated are given in TABLE 1. Included also are average levels obtained by Yavorsky *et al.*³ from human tissues at autopsy, which are not saturation levels. Simultaneous determinations by Yavorsky *et al.*³ on guinea pig tissues using dichlorophenol-indophenol titration procedure gave levels

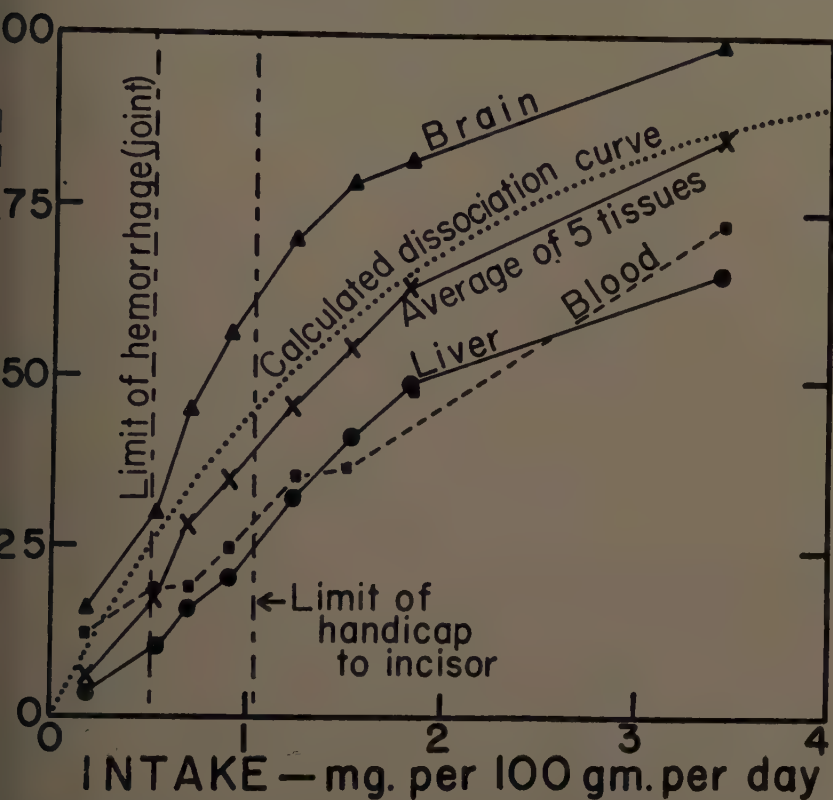


FIGURE 1. Relationship between ascorbic acid intake and ascorbic acid concentration of guinea pig tissues. Reproduced by permission of *Physiological Reviews*.¹

TABLE 1
LEVELS OF ASCORBIC ACID IN VARIOUS TISSUES

Tissue	Guinea pig ceiling levels* (mg. %)	Human autopsy levels† (mg. %)
Brain	19	30
Liver	24	12
Spleen	43	13
Kidney	9	10
Heart	7.5	5
Adrenal	119	55

*Guether *et al.*²
†Yavorsky *et al.*³

similar to those for human tissue. Data of Bessey and King⁴ and of Ingalls⁵ on infant liver obtained at autopsy have shown that human liver ascorbic acid varies quantitatively, as does guinea pig liver, when the ascorbic acid of the diet changes from low to abundant amounts. These data all suggest that human and guinea pig tissues have similar ceiling or saturation levels.

If the relation of tissue ascorbic acid to intake is known then the relation of health to tissue levels and that of tissue levels to optimal nutrition remain to be assessed. It is interesting that ceiling values for guinea pig tissues approach the levels found in 40 species that synthesize their own ascorbic acid. This fact is often cited as justification for providing sufficient ascorbic acid for human beings to saturate the tissues. For liver of 40 species an average value of 23 mg. per cent (range 18 to 40) is obtained;⁶ for guinea pig liver from animals on a cabbage diet, 28 mg. per cent; from animals showing minimum signs of deficiency, 6 mg. per cent; and, from severely scorbutic guinea pigs, 0.8 mg. per cent.² In the guinea pig the mildest signs of ascorbic acid deficiency are defects in the developing incisors. These appear when the tissue concentrations fall below 40 per cent of the maximum (FIGURE 1). Scurvy does not develop until the tissues are less than 20 per cent saturated. The time required for the onset of scurvy in both the guinea pig and the human being is longer than that required for marked depletion of normal vitamin reserves.

In a study on 100 human beings Linghorne *et al.*⁷ observed a disadvantage to the gingival tissues with 10 or 25 mg. of dietary ascorbic acid as compared to 75 mg. per day. Others have found no gingival disadvantage at the dietary level of 10 to 25 mg. Part of this disagreement may be explained by the idea that once gingivitis becomes established it is maintained by local infection rather than by ascorbic acid deficiency. It is generally agreed, however, that symptoms of frank scurvy do not appear until the intake has fallen below 10 mg. per day and that the tissue concentrations are very low indeed.

Chevillard and Hamon⁸ have found that the ascorbic acid content of white blood cells and platelets of the guinea pig closely parallels the content of the tissues. Consequently the white cells would be a valid measure of tissue saturation. Tournay *et al.*⁹ have shown that the relationships between white blood cell and plasma levels of human beings and guinea pigs are similar. Thus at high concentrations in plasma the white blood cells concentrations of both species are 25 to 30 mg. per cent. At low plasma levels, the white cell concentration decreases sharply. Levels below 20 mg. per cent indicate deficiencies. The white cells thus offer an indirect measure of tissue levels in human studies.

Ascorbic Acid in Human Beings

The chemical means of estimating tissue levels and nutritional status for ascorbic acid of human beings include measurement of the vitamin in plasma, whole blood, white blood cells, platelets, and urine. An advance in methodology that greatly facilitated such measurements was made by Bessey and Lowry.¹⁰⁻¹² These investigators provided techniques and proved the usefulness of these methods for ascorbic acid determinations in 10 cu. mm. of serum and in white cells and platelets from 0.1 ml. of blood that could be obtained from

finger of an adult or from the toe of an infant. Measurement could also be made on 25 to 100 cu. mm. of urine quite rapidly. The colorimetric methods are based on the reaction of dehydroascorbic acid and diketogulonic acid with 2,4-dinitrophenylhydrazine modified to improve precision and to simplify the procedure. An extensive study was carried out on 103 healthy volunteers in the Royal Canadian Air Force.¹³ Three groups of men received controlled levels of ascorbic acid, 8, 23, or 78 mg. per day for 8 months. The white blood cell and platelet levels (FIGURE 2) bear an almost straight-line

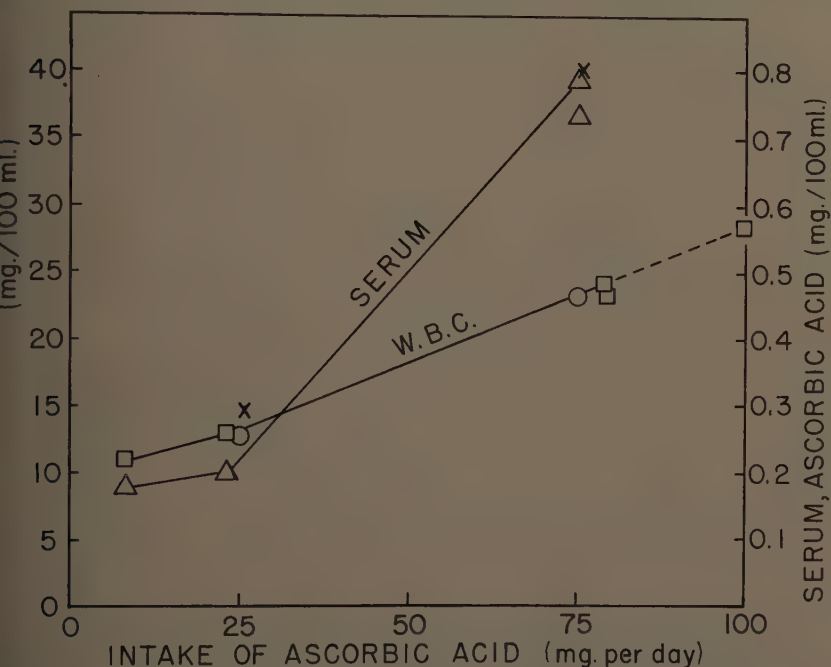


FIGURE 2. Relation of white blood cell (plus platelets) and serum ascorbic acid to intake in human beings. Levels of ascorbic acid in white blood cells and in serum represented by Δ and \times , respectively, are average figures from Lowry *et al.*;¹³ by \circ and \times from Davey *et*

relationship to the intake of ascorbic acid. At 78 mg. per day the white cells are not saturated. The dotted line represents the continuation to the saturation level. Data from Davey *et al.*¹⁴ fall very close to the same line. The serum levels do not follow a linear relationship with intake and are not a straight-line function of the white cell ascorbic acid level. With serum values below 0.4 mg. per cent, white cell ascorbic acid is diminished. The average white cell and serum concentrations parallel each other in this range, but individual correlation is poor.

Since the white blood cells are a good measure of total body ascorbic acid, the same relationships must hold between serum and total body ascorbic acid as between serum and white cells. A direct proportionality between white cell

and total body ascorbic acid has been established for the guinea pig. There is indirect evidence that this relationship holds for the human being as well. During realimentation of volunteers¹³ depleted of ascorbic acid, the rise in white blood cell ascorbic acid was parallel to the amount of the vitamin retained in the body.

Various estimations by different means have been made of total body ascorbic acid for a 70-kg. man. From the study of Crandon *et al.*¹⁵ 4 to 6 gm. were required to realiment a scorbutic person. From the Ingalls' analysis on human liver,⁵ Lowry calculated that 3 to 4 gm. were needed for saturation. From simultaneous measurement of tissue deficit and white blood cell levels of 14 persons who had received 8 and 23 mg. of ascorbic acid per day, the maximal total body content was estimated at about 4 gm. From studies on patients given L-ascorbic acid-1-C¹⁴, Hellman and Burns¹⁶ estimated 1.5 gm. as the body pool. These patients were obviously not healthy individuals and, according to serum and urinary levels, were far from saturated with ascorbic acid.

TABLE 2
ESTIMATION OF 24-HOUR EXCRETION FROM PLASMA LEVELS BELOW 1.4 MG. %

Level of ascorbic acid in 3 patients		
Plasma* (mg. %)	Urinary excretion (mg./24 hours)	
	Calculated	Found*
0.35	7.0	14
0.54	10.8	11
1.00	20.0	20

* Data from Hellman and Burns.¹⁶

acid. In the presence of a wasting disease, the liver is known to shrink greatly, perhaps to one third or one half of its normal size; other tissues also shrink. Thus these patients have a lower body pool than normal individuals. Low levels of ascorbic acid in white cells have been found in cancer patients and in chronic noncancer disease, indicative of low body stores.^{17,18} It would be desirable to have accurate measures of the body pool for normal human beings using isotopes. Until such information is obtained the first three calculations (TABLE 2) of the body pool of persons saturated with ascorbic acid give an indication of optimal body stores. The body pool of ascorbic acid for guinea pigs using C¹⁴-tagged ascorbic acid was calculated by Hellman and Burns as 54 mg./kg.¹⁶ This is very close to the quantity 50 mg./kg. estimated for saturation of human tissues.

The urinary excretion of ascorbic acid is very interesting, as illustrated by FIGURE 3. Friedman *et al.*¹⁹ and many others have described the threshold phenomenon quite clearly. With plasma levels below 1.4 mg. per cent, the plasma clearance is about 1.5 mg./min. or 2 l./24 hours. In this range the average 24-hour excretion will equal the plasma level in milligram per cent \times 20. As the plasma level rises above 1.4 mg. per cent, the clearance reaches

ml./min. at 2 mg. per cent and approaches glomerular filtration rate at 10 mg. per cent. The urine levels (TABLE 2) calculated from plasma levels of patients of Hellman and Burns illustrate how closely the calculated values approximate actual determinations in urine.

Ascorbic acid in white cells, serum, and urine of 4 persons continually saturated with ascorbic acid for 98 days are shown in TABLE 3.²⁰ The daily dose of 1000 mg. did not increase the white cell level above 30 mg. per cent or the

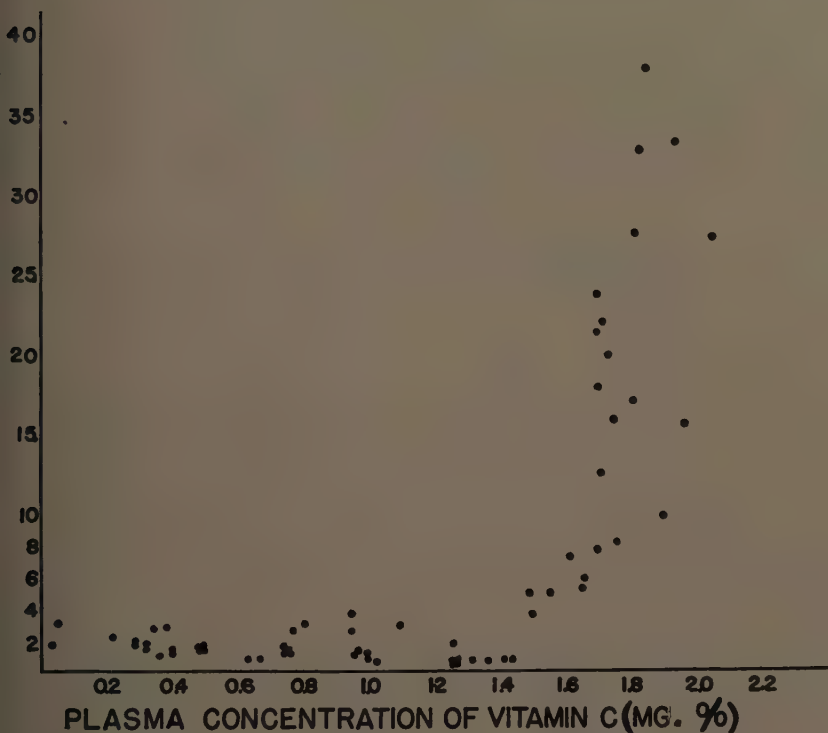


FIGURE 3. Relationship between vitamin C clearance and plasma concentration of vitamin C. Reproduced by permission of the *Journal of Clinical Investigation*.¹⁹

TABLE 3
ASCORBIC ACID IN URINE, WHITE BLOOD CELLS, AND SERUM OF FOUR PEOPLE
RECEIVING 1000 MG. PER DAY FOR 98 DAYS*

Day	Urine (mg./day)	WBC (mg. %)	Serum (mg. %)
0	—	27	1.22
5	817	28	1.81
21	804	30	1.79
39	714	28	1.82
98	822	28	1.64

Data from Lowry *et al.*²⁰

serum level above 1.8 mg. per cent. About 80 per cent of this dose was excreted. With large doses of ascorbic acid, the recovery in urine is 60 to 80 per cent, a remarkably high return. This and the favorable renal threshold permit virtual titration of the degree of unsaturation with regard to this vitamin. If a sufficiently large amount of ascorbic acid is given, the retention in the body (with suitable correction for destruction) should equal the tissue "deficit." The vitamin is best given in divided doses to avoid flooding of the blood stream.

Data (TABLE 4) from Lowry *et al.*¹³ illustrate determination of the tissue deficit of 4 persons, who, after receiving 8 mg. per day for 8 months were each given 2000 mg. in 10 hourly doses. It is assumed that there was no retention on the last day and that the difference between the excretion on the last day and the preceding days was equal to the amount retained, 2223 mg. Thus the extent of tissue depletion was measured by determination of the amount

TABLE 4
MEASUREMENT OF TISSUE "DEFICIT" OF ASCORBIC ACID*

Day	Urinary		Total retained (mg.)
	Excretion (mg.)	Retention (mg.)	
1	11	1528	2223
2	980	659	
3	1430	136	
4	1539	None	

* Data from Lowry *et al.*¹³ on 4 persons who had an intake of 8 mg. of ascorbic acid per day for 8 months. They were given 2000 mg. per day in 10 hourly doses; the retention was calculated by difference from excretion on the last day.

of ascorbic acid necessary to restore the concentration in white blood cells and tissues of the body as a whole to maximum values. This measurement is more time-consuming than the usual load test but gives more exact information. It is not subject to the errors that may arise in the load test if an amount of ascorbic acid much lower than the tissue deficit is given. When such a load dose is given, there may be no excretion or, if the dose is given rapidly, spilling into the urine may occur due to flooding of the blood stream.

The excretion of a metabolic product of ascorbic acid or of the product of some enzyme activity specifically affected by its concentration might be presumed to offer a means of assessing nutritional status in human beings. Thus far studies designed to find such excretion products have not been rewarding. The principal route of excretion of C¹⁴-tagged ascorbic acid in human beings is the urine.¹⁶ Only 5 per cent of the dose was found in the respiratory CO₂. About 42 per cent of the radio-carbon is excreted in urine. Of this amount, 4 per cent is excreted as oxalate; 20 per cent as ascorbic acid; 20 per cent as dikeogulonic acid; and 2 per cent as dehydroascorbic acid. Thus conversion of ascorbic acid to oxalate may account for the major part of the urinary oxalate excreted by man. It is interesting that conversion of ascorbic acid to oxalate

and not increase at an intake of 4 gm. of ascorbic acid per day.²¹ Rapid urinary excretion of the vitamin as described above could account for this limited conversion to oxalate. In contrast to that in the human being, the chief metabolic product of L-ascorbic acid-1-C¹⁴ in the guinea pig is respiratory CO₂.²² Only a small fraction is excreted as oxalate in the guinea pig.

For dietary surveys designed to measure the total intake of ascorbic acid, determinations might be made on the basis of urinary excretions. Urinary output of ascorbic acid based on creatinine excretion of random urine specimens could give a valid measure of ascorbic acid consumption if the known physiology of this vitamin is considered. For nutritional survey purposes, serum levels are useful, as illustrated in TABLE 5.²³ In these population groups many persons with serum levels below 0.4 mg. per cent were found indicating a poor state of ascorbic acid nutrition. Measurements of white cell levels or of tissue deficits would have given more precise information on these suboptimal groups.

TABLE 5
SERUM ASCORBIC ACID IN POPULATION GROUPS

Place	No. of persons	Percentage of persons with ascorbic acid*	
		Below 0.4 mg. %	Above 0.7 mg. %
Manila, Philippines	202	53	17
New York, N.Y., poor-area school	150	48	27
Eight high schools	1000	29	45
Greenland	370	59	—

* Data from Burch *et al.*²³

Summary

For the chemical estimation of ascorbic acid status the measurement of one parameter will not suffice. A combination of measurements of serum levels, urinary excretion, white cell level, and tissue deficit will effectively determine the range of ascorbic acid nutrition from the state of abundant supply to severe deficiency. Thus:

(1) If white blood cells are 27 to 30 mg. per cent, the tissues are saturated; the body will contain about 50 mg. of ascorbic acid per kilogram; urinary excretion will effectively measure intake with 60 to 80 per cent recovery of a large dose; serum levels will be above 1 mg. per cent and will indicate a surely adequate intake. An intake of 100 mg. per day or above is shown by these criteria.

(2) If the white blood cells and tissues are somewhat less than saturated, then urinary excretion will vary from 20 to 60 per cent recovery of the amount ingested, and the plasma ascorbic acid will range from 0.4 to 1 mg. per cent. An intake of 40 to 100 mg. per day is indicated.

(3) If the white cells and tissues have fallen to 50 per cent of saturation, the urinary excretion is very low. The plasma levels are so low that they are not an accurate measure of status. An intake of 10 to 15 mg. per day is indicated.

(4) Likewise if the white cells are 25 per cent saturated, an intake of 5 to 7 mg. per day is indicated. An alternate measure of the tissue deficit by the administration of large divided doses of ascorbic acid will be very informative.

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APPRAISAL OF METHODS FOR THE DETERMINATION OF L-ASCORBIC ACID

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Ascorbic acid methodology may be considered under two general headings: (1) oxidation-reduction procedures, with indophenol as an example of the reagent used; and (2) procedures based on treatment of the chromogen formed by coupling oxidized ascorbic acid with 2,4-dinitrophenylhydrazine.

Oxidation-Reduction Methods

In these methods, oxidation of the two loosely-bonded hydrogen atoms of the dienolic group of ascorbic acid is measured. Methods have been proposed that determine this compound by measuring its oxidation with iodine,¹ phosphomolybdate,² ferric chloride,³ methylene blue,⁴ 2,6-dichlorophenolindophenol,⁵ and other substances.

The most widely used oxidizing reagent is 2,6-dichlorophenolindophenol, introduced by Tillmans *et al.*⁵ in 1932. Use of this reagent appears to permit the development of the greatest precision and specificity obtainable with any of the oxidation-reduction reagents proposed. Bessey⁶ showed that the highest degree of specificity is obtained by carrying out the reaction at pH 3.5. At a pH of approximately 1, indophenol is reduced by the hydrogen ions of the reacting fluid, and at pH values above 4, some naturally-occurring compounds, for example, substances containing phenolic or sulfhydryl groups, react with indophenol. Considerable success has been obtained with photometric techniques that permit correction for compounds with reducing groups that react with indophenol more slowly than with ascorbic acid. However, there is no satisfactory adaptation to avoid the interference of compounds such as thiosulfate, sulfite, and ferrous, cuprous, or stannous salts, which react rapidly with indophenol.

Visual titration techniques, using indophenol reagent, are limited by the difficulty of judging end points. The greatest precision and specificity are obtained by photometric measurement of the difference between the optical densities of the reagent before and after addition of an extract containing ascorbic acid. Photometric methods for the determination of ascorbic acid by the use of 2,6-dichlorophenolindophenol have been developed by Evelyn *et al.*,⁷ Lindlin and Butler,⁸ Bessey,⁶ Loeffler and Ponting,⁹ Hochberg *et al.*,¹⁰ and others. In our laboratory the greatest precision and specificity have been obtained by an adaptation¹¹ of the method of Evelyn *et al.*,⁷ the indophenol reagent being buffered to yield a pH of approximately 3.5, when the reagent and ascorbic acid solution are mixed.

Photometric methods have the following advantages in comparison with visual titration procedures: (1) the inaccuracy due to difficulty in judging a rapidly disappearing end point is eliminated; (2) the effect of turbidity or pigmentation in the unknown mixture can be overcome to a considerable extent by adjustment of the colorimeter to compensate for the extraneous substances;

(3) correction can be made for slowly reacting, nonascorbic acid-reducing substances by taking timed successive readings of the optical density and by extrapolating back to zero time; (4) greater sensitivity and precision in low concentrations of ascorbic acid are obtainable.

Possibilities and limitations. The possibilities and limitations of oxidation-reduction methods have been examined by Chapman *et al.*¹² The data of these authors are shown in TABLE 1. They used seven different modifications of the indophenol technique for making comparative studies of multivitamin pharmaceutical products. Serious errors were observed in analyzing preparations that contained ferrous sulfate, cuprous chloride, and cupric sulfate. The high values were due to the reduction of indophenol by ferrous or cuprous ions. In certain procedures where conditions were adapted to prevent this effect, correct values were obtained. With three of the procedures, not adapted to overcome the oxidant effect of the cupric ion, low values were obtained. With

TABLE 1
EFFECT OF ADDED SUBSTANCES ON RECOVERY OF ASCORBIC ACID
AS DETERMINED BY EIGHT METHODS

Method of analysis	Substances added*					
	Ferrous sulfate	Cuprous chloride	Cupric sulfate	FeSO ₄ + CuSO ₄	Liver fraction	Cod liver oil
Roe <i>et al.</i>	100.3	101.2	101.2	101.2	100.0	100.0
Huelin and Stephens, using H ₂ O ₂	9.2	71.9	92.7	9.2	105.5	98.2
U.S.P., XIV	378.8	89.5	98.5	248.2	106.9	99.2
Gawron and Berg	97.9	75.5	65.7	38.0	101.7	97.3
Watanabe, using 5% (COOH) ₂	146.4	97.1	100.6	234.7	108.2	99.4
Frosst, using 2.5% H ₂ SO ₄	99.7	99.5	100.6	88.8	112.4	98.5
Brown and Adam	98.7	211.5	43.2	37.3	105.6	98.9
Robinson and Stotz, using H ₂ O ₂	82.8	87.4	87.5	76.5	107.7	102.5

* Per cent recoveries. Added at rate of 1 gm. to 100 mg. of ascorbic acid. Reproduced by permission of *Analytical Chemistry*.¹²

the two methods using H₂O₂, introduced by Robinson and Stotz¹³ to minimize the interference due to reduced tin and iron, sulfite, and reductones, low values were obtained, apparently due to the oxidant effect of H₂O₂. Correct recoveries in all instances were obtained when the 2,4-dinitrophenylhydrazine method¹⁴ was used for analysis of the same pharmaceutical preparations, as shown in line 1 of TABLE 1.

Techniques based on the oxidation-reduction principle, especially the indophenol procedures, are rapid, practicable, and hence useful. In using these procedures the analyst must make sure that nonascorbic acid-reducing substances are not present in the solution examined in objectionable amounts, and that the vitamin exists totally, or almost so, in the reduced form.

Establishment of specificity. It has been necessary to establish the specificity of chemical methods for the determination of ascorbic acid by chemical procedures, since bioassay methods are incapable of achieving satisfactory quantitative results. The specificity of chemical methods for ascorbic acid was studied in 1944 by Roe and Oesterling.¹⁵ These authors made comparative

analyses on the same tissue extract, using an indophenol photometric method and the dinitrophenylhydrazine procedure. It was reasoned that obtaining essentially the same values in analyses by two methods, completely dissimilar chemical principles, would be strong evidence that each method is specific for the determination of ascorbic acid in the extract analyzed. Close agreement between most of the values by the two methods was observed. Where differences beyond experimental error were found, they were accounted for, completely in some instances, and partially in others, by the direct analysis for dehydroascorbic acid and diketogulonic acid.¹⁵ These results suggested that both the indophenol and the dinitrophenylhydrazine methods are satisfactory for the determination of ascorbic acid in plant tissues; and that if doubt exists concerning the specificity of either method, the procedure for performing comparative analyses by the two completely dissimilar chemical methods would be useful. The applicability of the indophenol methods, however, is limited to tissues in which oxidation of ascorbic acid has not occurred to a significant extent.

In considering the use of oxidation-reduction methods in animal tissues, it must be decided whether the small amounts of the oxidation products of the vitamin not estimated by these methods would alter an interpretation of the results. Dehydroascorbic acid and diketogulonic acid were found in the tissues of well-fed and scorbutic guinea pigs by Damron *et al.*,¹⁶ who used the differential dinitrophenylhydrazine method.¹⁷ The values found were small, but they must be accepted as true findings since they were obtained by analytical methods in which the tissues were extracted by a strong reducing solution (10 per cent SnCl_2 in 5 per cent HPO_3 solution).

Dinitrophenylhydrazine Methods

Principles and specificity. The dinitrophenylhydrazine procedures are based on the photometric measurement of the color produced by treatment of the chromogen formed by the coupling of 2,4-dinitrophenylhydrazine with the phenolic groups of oxidized ascorbic acid.¹⁴ This principle has been applied to the development of methods for the determination of ascorbic acid, dehydroascorbic acid, and diketogulonic acid.¹⁷

Ascorbic acid must be oxidized to dehydroascorbic acid before a coupling with 2,4-dinitrophenylhydrazine can occur. The best oxidizing reagent is nitrite, which serves the dual function of acting both as an oxidant and as a clarifying agent. Acetic, trichloroacetic, or oxalic acid must be present in the reaction fluid to prevent adsorption of the dehydroascorbic acid upon the nitrite. Bolin and Book¹⁸ proposed the use of indophenol as an oxidant in this procedure. This reagent has no advantage as an oxidant, and it has the serious objection of not providing for the clarification of the tissue extract. In addition to clarification of extracts, giving colorless filtrates, Norit also removes interfering substances that may be present in tissue extracts oxidized by either reagent, for example, by bromine, as shown by Mills and Roe.¹⁹

The specificity of the dinitrophenylhydrazine method is based upon the following principles: (1) color is produced only with 2,4-dinitrophenylhydrazine derivatives of 6-carbon and 5-carbon sugarlike compounds; (2) the rate of

coupling with dehydroascorbic acid is much faster than with sugars or sugar-like compounds; (3) measurable chromogen formation from nonascorbic acid substances is avoided by dilution of the extract and by carrying out the coupling reaction at a low temperature, 37° C.; (4) the coupling reaction takes place in a reducing medium produced by such reducing reagents as thiourea or stannous chloride.¹⁹

Temperature of the coupling reaction. It is very important that the coupling reaction in this method be carried out at moderate temperatures. Roe and Kuether,¹⁴ using a temperature of 37° C., called attention to the fact that the coupling of sugars with 2,4-dinitrophenylhydrazine is very slow at this temperature and does not result in interference from the amounts of sugar found in plant and animal tissues when required dilutions of the extract are used.

The proposal of Schaffert and Kingsley²⁰ to carry out the coupling reaction in this method at 100° C. results in erroneously increased values. The errors that may result from this modification were shown by studies directed toward re-examining the importance of the temperature at which the coupling reaction is carried out. Twelve common food substances were homogenized or diluted in 0.5 per cent oxalic acid, and 4 animal tissues were ground under 4 per cent trichloroacetic acid. One part of tissue was extracted with 50 volumes of acid solution, except muscle, which was ground with 10 volumes of extractant. The extracts were treated with Norit. Four ml. aliquots of each extract and of a dehydroascorbic acid standard were placed in colorimeter tubes, and 1 ml. of 2,4-dinitrophenylhydrazine reagent* was added to each tube. Duplicate tubes containing aliquots from each extract, and from the dehydroascorbic acid standard, were submitted to the following conditions: (1) one group was placed in a water bath at 37° C. for 3 hours; (2) another group was boiled in a water bath for 10 min.; (3) a third group was placed in a refrigerator at 15° C. for 17 hours. At the completion of the treatment, 5 ml. of 85 per cent H₂SO₄ were added, dropwise, to each tube in an ice bath. After standing for 30 min., color comparison was made. Excellent agreement between the results obtained with the coupling reaction at 37° C. and 15° C. was observed, except in the tomato, which showed a value of 18 per cent higher at 37° C. The values at 37° C. are considered true findings for ascorbic acid, except for the tomato.

The values found at 100° C. were much higher than those observed with the coupling reaction at 15° C., except in two instances, kale and spleen. Notable examples of higher values in plant tissues were carrots, squash, pineapple, cucumber, and tomato, which showed values higher than those found at 15° C. by 145, 75, 78, 144, and 129 per cent, respectively. In the animal tissues the values at 100° C. for liver, kidney, and muscle were 35, 33, and 40 per cent higher, respectively, than the values obtained at 15° C. The higher values observed at 100° C. with plant and animal tissues are erroneous, as will be shown by the following discussion.

Coupling reaction rates involving substances that have different chemical structures are different; hence, it is possible to use a temperature at which the substance showing the faster reaction rate will yield measurable chromogen.

* Prepared by dissolving 2 gm. of 2,4-dinitrophenylhydrazine and 4 gm. of thiourea in 100 ml. of 9 N H₂SO₄.

and the compound that couples more slowly will not yield an appreciable amount of product. This principle offers a solution to the problem of interfering substances in the determination of ascorbic acid. If interfering substances that couple slowly with dinitrophenylhydrazine are present in the tissue extracts, they would be expected to yield more chromogen at 37° C. than at 100° C. As the results obtained with the coupling reaction at 15° C. and 37° C. are the same, within the limits of experimental error, measurable amounts of nondehydroascorbic acid chromogen were not formed (except in the tomato), and the results obtained at 37° C. are therefore considered specific for ascorbic acid.

INTERFERENCE IN THE DINITROPHENYLHYDRAZINE METHOD

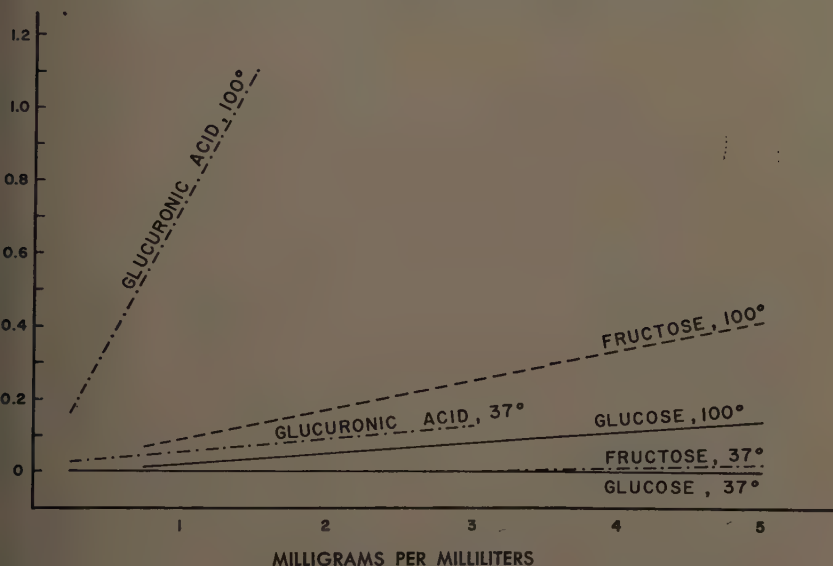


FIGURE 1. Increase in optical density in the dinitrophenylhydrazine method from glucose, fructose, and glucuronic acid at 37° C. and 100° C.

The higher values found at 100° C. are attributed to the formation of additional chromogen from nondehydroascorbic acid substances that react appreciably with dinitrophenylhydrazine at this temperature.

Measurement of possible interference. The interfering substances in plant and animal tissues that may cause additive errors in the dinitrophenylhydrazine method are principally glucose, fructose, glucuronic acid, or glycogen. The amount of interference that may be expected from glucose, fructose, and glucuronic acid at 100° C. and 37° C. has been determined and is shown in Figure 1. At levels ranging from 0.75 mg. to 5 mg. per ml. glucose showed an increase in optical density at 37° C.; at 100° C. the optical-density increase at the same concentrations of glucose ranged from 0.031 to 0.109. At 37° C., fructose showed an optical-density increase of 0.019 in concentrations of 5 mg. per ml. and no increase with concentrations of 3 mg. per ml. or lower; at 100°

C., fructose yielded optical-density increases of 0.066 to 0.412 in concentrations of 0.75 mg. to 5 mg. per ml., respectively. Free glucuronic acid, when coupled with 2,4-dinitrophenylhydrazine at 37° C., yielded increases in optical density of 0.022 to 0.125 with concentrations of 0.25 to 3.0 mg. per ml.; and at 100° C., this compound gave optical density increases of 0.157, 0.466, and 1.103 with concentrations of 0.25, 0.75, and 1.5 mg. per ml. respectively. In all instances the curves of FIGURE 1 show marked increases in optical density where the temperature of the reaction mixture was raised to 100° C.

These data show that, in the dinitrophenylhydrazine procedure, interference from glucose, fructose, glycogen, or glucuronic acid, will not occur under the conditions recommended by Roe and Kuether.¹⁴ With the temperature of the coupling reaction set at 37° C. and a dilution of the extract of 1:10, no interference results in the analysis of tissues containing 5 per cent of glucose or glycogen, or 3 per cent of fructose. Since in the regular procedure a dilution of 1:50 or greater is recommended, it is clear that interference from glucose or fructose does not occur unless large amounts of sugar are added, as in the processing of certain foods. In the latter instance the dilution of extracts necessary for the analysis can be calculated from FIGURE 1.

Glucuronic acid is present in tissues or body fluids principally in the conjugated form. The latter will not couple with 2,4-dinitrophenylhydrazine until after hydrolysis, which is slight at 37° C. From the data presented, it can be assumed that interference in the dinitrophenylhydrazine method from glucuronic acid in tissues either does not occur at all, or if present under exceptional circumstances where high glucuronate levels are encountered, would be very small.

Analysis in presence of reductones and reductic acid. Heating sugars in a mildly alkaline medium produces compounds called reductones and reductic acid. Mapson²¹ found these substances in certain preserved and cooked foods and in foods prepared at temperatures high enough to cause a scorching of the material. These substances interfere in both the dinitrophenylhydrazine method and in the usual oxidation-reduction procedures. A good solution of this problem is to separate the components of the extract by paper chromatography and then to determine the ascorbic acid, or other substances of interest, after identification and elution. A chromatographic procedure well suited to this problem was published by Chen *et al.*²²

Proposal. Based upon the results obtained, the procedure for performing comparative analyses with the temperature of the coupling reaction set at 15° C. and 37° C., or at other relatively low temperatures separated by 20 or more degrees, is proposed as a method for determining the specificity of the dinitrophenylhydrazine method for ascorbic acid.

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THE ESTIMATION OF DEHYDRO-L-ASCORBIC ACID WHEN PRESENT IN LOW CONCENTRATION IN TISSUES, BY THE ROE AND KUETHER PROCEDURE

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The Roe and Kuether method¹ for the estimation of dehydro-L-ascorbic acid (DHA) in tissues has been modified in our laboratory to permit the estimation of this acid when present in low concentrations. The normal procedure with many plant tissues yields extracts that contain less than 0.25 to 0.5 $\mu\text{g./ml.}$ after dilution with the phenylhydrazine reagents; even lower values are encountered if the tissue is exposed to anaerobic or partially anaerobic conditions before extraction. Moreover many metaphosphoric acid extracts of plant tissues when treated with 85 per cent H_2SO_4 , as in the Roe and Kuether method, develop brown colors on standing that absorb in the 530-m μ region, making it impossible to estimate the concentration of the hydrazone formed from DHA with any accuracy.

The bis(dinitrophenyl)hydrazone formed from DHA is virtually insoluble in the sulfuric-phenylhydrazine reagent, and may be collected quantitatively from large volumes of extract by centrifugation and/or filtration. In this way the hydrazone may be concentrated and, at the same time, separated from other undesirable constituents of the tissue, thus increasing the accuracy of the estimation.

The preliminary procedure of Roe and Kuether is followed through to the stage of incubation of the extract with the phenylhydrazine reagents. Since we were attempting to estimate small amounts of DHA in the presence of a large amount of ascorbic acid, we found it advisable (although thiourea was present) to remove with nitrogen all oxygen from the solution before incubating the extract in order to prevent any possible oxidation of ascorbic acid.

After incubation for 3 hours at 37° C., the solution is centrifuged at 20,000 g for 30 min. to aggregate the insoluble hydrazones. These are collected by filtration through sintered glass filters, and the collected precipitate washed with 0.1 N H_2SO_4 to remove the unchanged 2,4-dinitrophenylhydrazine. The washed hydrazones are dissolved in a small volume of ethyl acetate, and the solution transferred to a separating funnel. If, as in many plant extracts, hydrazones of the α -oxo acids are also present, these may with advantage be removed at this stage by extracting the ethyl acetate solution twice with a saturated solution of NaHCO_3 . By this treatment these α -oxo acid hydrazones dissolve in the aqueous phase, while the hydrazone derivative of DHA remains in the nonaqueous phase.

The ethyl acetate solution is washed again with 0.1 N H_2SO_4 . After careful separation, the nonaqueous phase is concentrated *in vacuo* to dryness. The hydrazones are taken up in 1 ml. of ethyl acetate to which is added 5 ml. of H_2SO_4 50 per cent w/v. After standing for 30 to 60 min. at room temperature the concentration of the hydrazone is determined from the difference between the absorption at 530 m μ of this solution and a similar solution in which the

HA had been previously reduced by H_2S in the usual Roe and Kuether method. This adaption of the original method has yielded consistent and reproducible results when referred to dehydroascorbic acid standards that have been carried through the same procedure.

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ROLE OF ASCORBIC ACID IN THE FORMATION AND MAINTENANCE OF DENTAL STRUCTURES

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Although many individuals of prehistorical and early historical periods probably experienced scurvy, one of the first individuals known to have described lesions certain to have been scurvy was Jacques De Vitry, Bishop of Acre, who described the disease that afflicted the Crusaders during their long journeys to the Holy Land. He writes "A sudden pain attacked their feet and legs; their teeth and gums were soon tainted with a kind of gangrene, and the sick could no longer eat; then the bones of their legs turned a horrible black and afterwards a great number of Christians after suffering grievous pains which they bore with great patience, went to rest in the bosom of the Lord, some lived till spring, then cured by the mild temperature."²³ In later years it is known that sailors became scorbutic while on long voyages and that one of their main complaints was swelling and hemorrhages of the gums and the loss of teeth.²³

Pathology of Dental Structures Due to Scurvy

Zilva and Wells²⁶ and Jackson and Moore¹⁸ were apparently the first individuals to examine dental structures of scorbutic animals microscopically. Zilva and Wells specifically noted fibrosis and the formation of osteoidlike deposits in the pulps of teeth, whereas Jackson and Moore described hemorrhages in them. Howe,¹⁷ Hojer,¹⁶ and Wolbach and Howe²⁴ induced scurvy in guinea pigs and made the now classic histological observations of the effect of scurvy on dental structures. They demonstrated that ascorbic acid is essential for dentinogenesis. They found that odontoblasts regressed to a cuboidal or squamous form and that dentin production failed in guinea pigs maintained on a prolonged scorbutogenic diet (compare FIGURES 1 and 2). During the development of the scorbutic state they noted that odontoblasts produced either a modified product designated osteodentin or a liquid substance that accumulated between the odontoblasts and dentin. They also noted an osteoporosis. Dentin and bone produced during the scorbutic state are easily differentiated from the normal by the Rinehart and alloxan-Schiff stains⁴ (FIGURES 3, 4, 5, and 6).

Kotanyi¹⁹ and, later, Boyle *et al.*¹⁻³ described alterations of ameloblasts and an enamel hypoplasia due to scurvy. Boyle attributed alterations of amelogenesis to traumatic injury to the formative end of the tooth secondary to the breakdown and loss of collagen in the periodontal membrane. FIGURE 2 illustrates a definite disorder of ameloblasts situated adjacent to the site that experienced a failure of dentinogenesis. Evidence of trauma to this area is lacking. FIGURES 7 and 8 illustrate the reactivity of organic enamel matrix and granules in normal functional ameloblasts and cells of the stratum intermedium

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with the Taenzer-Unna orcein stain. The granules are presumed to be pre-enamel because (1) they stain with orcein as does pre-enamel and the organic matrix of enamel; (2) ameloblasts are known to produce enamel; and (3) functional ameloblasts contain the granules, but differentiated nonfunctional ameloblasts do not. Scorbutic animals exhibit a markedly increased concentration of the granules in the stratum intermedium and a moderate increase in ameloblasts situated in the zone devoid of subjacent dentin; however, ameloblasts and cells of the stratum intermedium located over normal dentin and enamel

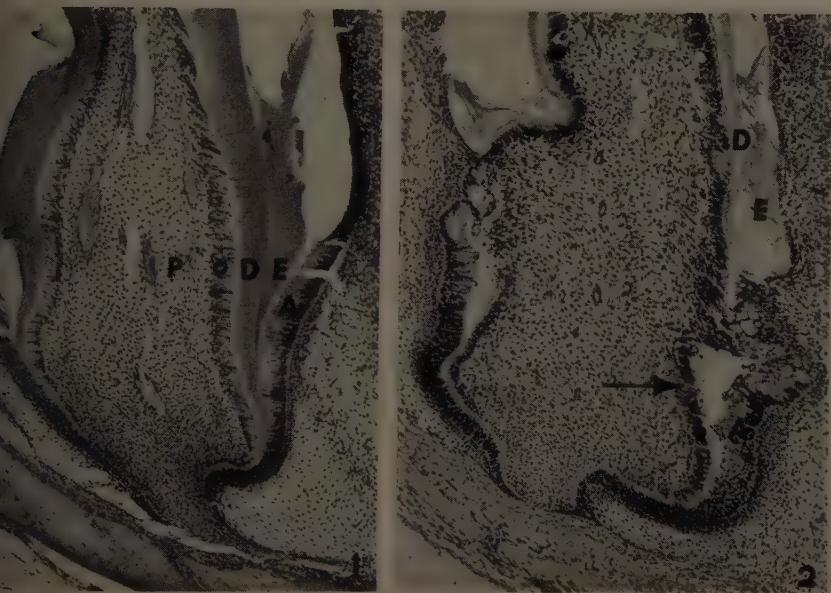


FIGURE 1. Photomicrograph of the growing apical end of a molar tooth from a guinea pig on a scorbutogenic diet for 2 weeks and then given 1 mg. vitamin C daily for 1 week. P, pulp; O, odontoblasts; E, enamel matrix; A, ameloblasts; D, dentin. H and E stain. $\times 63$.
FIGURE 2. Photomicrograph of the growing end of a molar tooth from a guinea pig on a scorbutogenic diet for 3 weeks. Note the failure of dentin production that should extend furcally. Ameloblasts and odontoblasts appear to differentiate normally, but odontoblasts degenerate and die (arrow) at the time dentinogenesis should begin. Alterations of ameloblasts are also noted in this zone. Loss of bone is evident. H and E stain. $\times 63$.

manifest no alterations (FIGURES 9, 10, and 11). The normal progression of odontogenesis over dentin in scorbutic guinea pigs and the pathological condition that ensues in areas without subjacent dentin reveal the dependence of ameloblasts upon a connective tissue.

Periodontal membranes of guinea pigs in advanced scurvy generally appear denser than normal, indicating some destruction of collagen, as Boyle has stated out.¹⁻³ Oxytalan fibers¹² in these areas sometimes appear partially degraded, but they usually appear unaffected (FIGURE 12). Tracer autoradiographical studies²⁵ revealed a greater than normal uptake of radioactivity in periodontal membranes, alveolar bones, and tooth pulps of scorbutic guinea

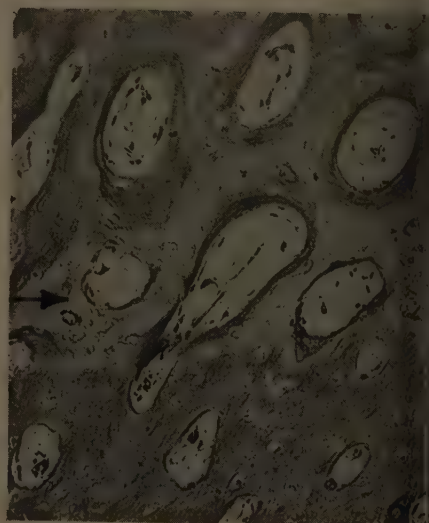
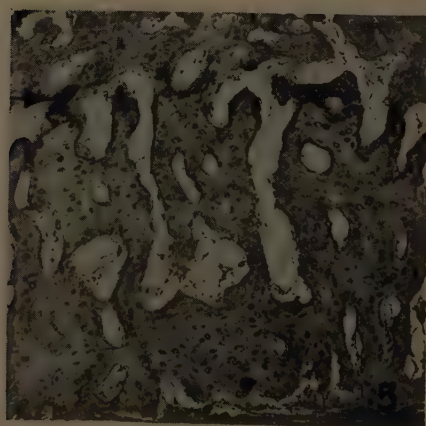


FIGURE 3. Photomicrograph near the growing end of a molar tooth from a guinea pig on a scorbutogenic diet for 3 weeks. Rinehart stain. Normal dentin (ND) stains red (*light*) abnormal dentin (AD), produced during the scorbutic state, stains blue (*dark*). $\times 63$.

FIGURE 4. Photomicrograph near the growing end of a molar tooth from a guinea pig on a scorbutogenic diet for 3 weeks. Alloxan-Schiff stain. Normal dentin (ND) stains pink abnormal dentin (AD), produced during the scorbutic state, remains unstained (*light*). $\times 63$.

FIGURE 5. Photomicrograph of a portion of the mandible of a guinea pig on a scorbutogenic diet for 2 weeks and then given 1 mg. vitamin C daily for 1 week. Rinehart stain. Normal bone produced during the scorbutic state (*below*) and after the scorbutic state (*above*), stains red (*light*). The dark band in the middle (*arrow*) is bone produced during the scorbutic state which stains blue (*dark*). $\times 63$.

FIGURE 6. Same as FIGURE 5. Alloxan-Schiff stain. Normal bone (*above and below*) stains pink (*dark*). The band of unstained bone in the center (*arrow*) was produced during the scorbutic state. Note the new bone lining the interior of the Haversian canal in the center of the photograph. $\times 63$.

gs after the administration of L-ascorbic acid 1-C¹⁴. The reader is referred to the papers of W. v.B. Robertson and B. S. Gould in this publication for discussions of the biochemical role of ascorbic acid in the formation and maintenance of collagen.

The above microscopic observations indicate that a characteristic feature of scurvy is a defect in the production and maintenance of intercellular substance. Ground substance is an essential component of intercellular substance, and its role in scurvy has been investigated by the use of tracer amounts of

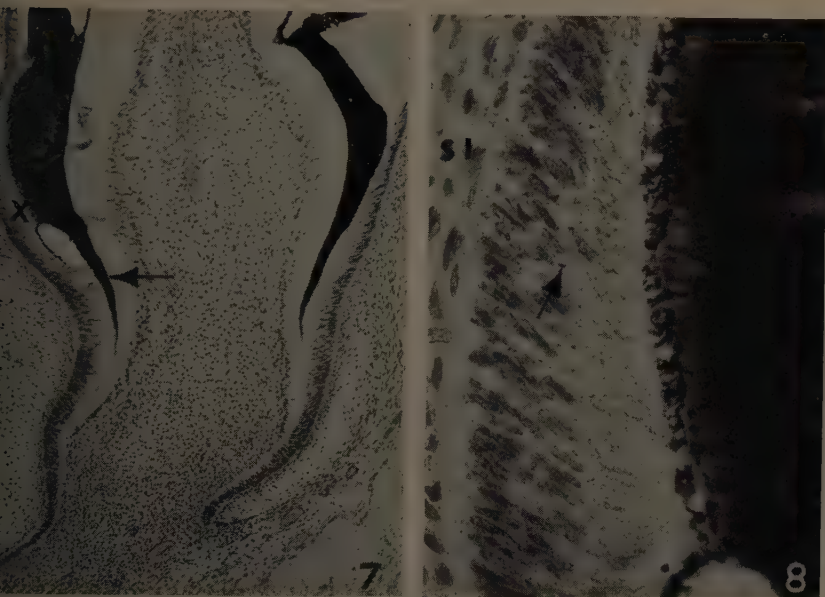


FIGURE 7. Same as FIGURE 1. Taenzer-Unna orcein stain. Enamel matrix (arrow) is intensely stained. $\times 63$.

FIGURE 8. Same as FIGURE 1. Magnification of the area marked X in FIGURE 7. Note nuclei (arrow), presumed to be pre-enamel, in ameloblasts and in the stratum intermedium zone. Taenzer-Unna orcein stain. $\times 435$.

labeled sulfur. Hill and Bourne found a retention of S³⁵ in cartilage cells,¹⁵ Reddi and Norstrom²¹ noted a decreased rate of synthesis of chondroitinuric acid. Friberg obtained an increase, no change, and a decreased amount of S³⁵ in various tissues including the teeth, depending upon the length of time the pigs were maintained on a scorbutogenic diet.¹¹ Friberg attributed the effect of scurvy on sulfate metabolism to inanition. It is apparent that more work is required to define precisely the role of ground substance in the etiology of scurvy.

Friberg noted a 25 to 50 per cent reduction of P³² incorporation into bones and teeth of scorbutic guinea pigs, and suggested that a suppression of mineralization occurs in scurvy.¹¹

Investigations with Nonvitamin C Antiscorbutic Compounds

Nonvitamin C compounds are being used to elucidate the mechanism of action of vitamin C. Useful indices of vitamin C activity in guinea pigs are

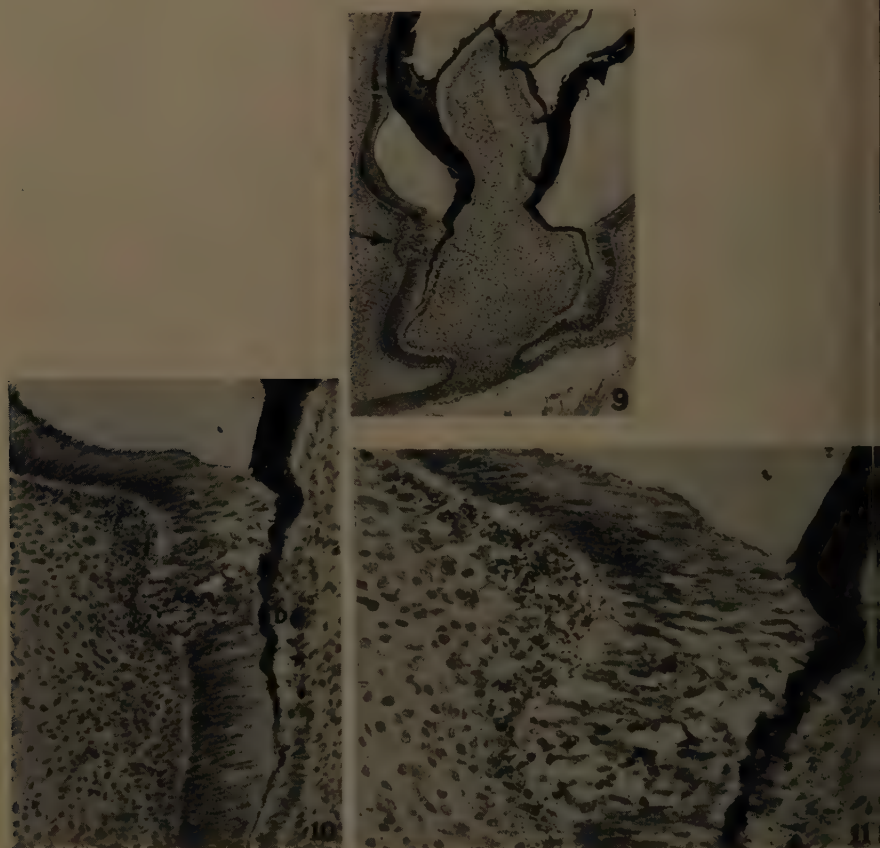


FIGURE 9. Same as FIGURE 2. Taenzer-Unna orcein stain. Note the alterations of amelogenesis (arrow). $\times 63$.

FIGURE 10. Same as FIGURE 9. Taenzer-Unna orcein stain. Magnification of the area indicated by the arrow in FIGURE 9. Note the degeneration of the odontoblasts (DO), the failure of dentin production, and the adjacent alterations of the ameloblasts (AA). Observe the greatly increased concentration of orceinophilic granules (presumably pre-enamel) in the stratum intermedium (SI), and the moderate increase of the granules in affected ameloblasts. $\times 205$.

FIGURE 11. Magnification of FIGURE 10. Taenzer-Unna orcein stain. $\times 435$.

a loss or gain of weight, survival rate, the reduction of serum alkaline phosphatase,²² the prevention of hemorrhages, a gerüstmark reaction in the epiphyses of long bones and dentinogenesis.⁷ We have administered various compounds to scorbutic guinea pigs and have achieved a separation of these pathoses, thereby suggesting multiple actions of vitamin C.²⁰

It has been shown previously that 3-methyl-1-ascorbic acid and 1-methyl-2-ascorbic acid have vitamin C action.^{10,13,14} L-Erythro-3-ketohexonic acid, D-lyxo-3-ketohexonic acid, L-lyxo-3-ketohexonic acid, and D-arabo-3-ketohexonic acid have been reported to be without vitamin C activity.^{8,10} D-Ascorbic acid has been reported to be without vitamin C activity in dosages of 100 mg. daily (20 mg. daily) that required to cure scurvy in guinea pigs with L-ascorbic acid.¹⁰ Dayton and Burns later demonstrated that D-ascorbic acid

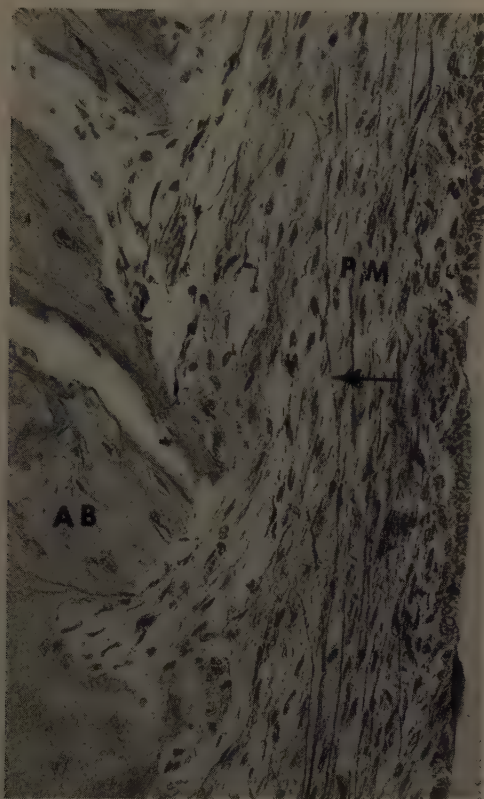


FIGURE 12. Photomicrograph of a periodontal membrane (PM) of a guinea pig on a vitamin C deficient diet for 3 weeks. Collagen is less dense than normal. Arrow points to a collagen fiber. AB, alveolar bone. Peracetic acid-aldehyde fuchsin-Halmi stain.¹³ $\times 240$.

metabolized and excreted at a far greater rate than is L-ascorbic acid, and on this basis suggested that the lack of vitamin C action may be due to an insufficient tissue concentration.⁹ The daily administration of 12 mg. of D-ascorbic acid to scorbutic guinea pigs did not prevent hemorrhages, but it did permit survival, a normal weight gain, and dentinogenesis.⁴

The effect of 3-methylcholanthrene on scorbutic guinea pigs was speculated to be of interest since it stimulates the production of ascorbic acid in rats.^{5,6} The daily administration of 10 mg. of 3-methylcholanthrene to scorbutic guinea pigs did not induce ascorbic acid biosynthesis, but it permitted normal dentino-

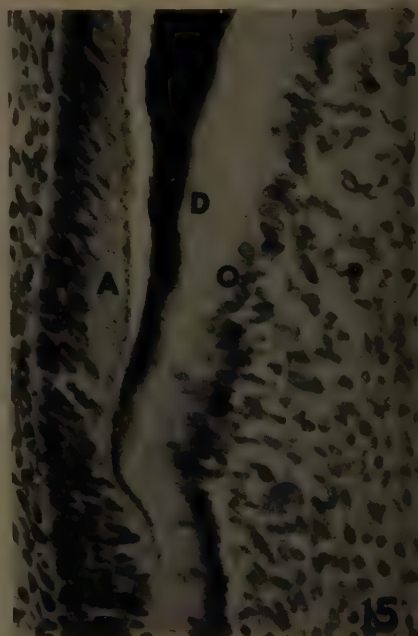


FIGURE 13. Same as FIGURE 1. Rinehart stain. $\times 63$.

FIGURE 14. Photomicrograph of the growing end of a molar tooth from a guinea pig on a scorbutogenic diet for 2 weeks and then given 10 mg. 3-methylcholanthrene daily for 1 week. Rinehart stain. Note the normal dentinogenesis and amelogenesis. $\times 63$.

FIGURE 15. Same as FIGURE 14. Taenzer-Unna orcein stain. Note the normal dentinogenesis and amelogenesis. $\times 285$.

esis, restored mucopolysaccharides in gingival tissues, and it largely prevented the hemorrhages; however, it did not prevent the typical gerüstmarktion in the epiphyses of long bones (FIGURES 13, 14, and 15).²⁰ 2,6-Dichlorophenolindolphenol, like ascorbic acid, prevented the substrate-induced inhibition of *p*-hydroxyphenylpyruvic acid oxidase, but it was without effect on dentinogenesis and hemorrhages in scorbutic guinea pigs.* The paper is referred to the paper by Bert N. La Du and Vincent G. Zannoni in a symposium for a detailed discussion on this reaction.

Thus some, but not all, of the effects of scurvy may be prevented by certain vitamin C compounds, and the reagent need not be structurally related to vitamin C. These findings are consistent with the concept that vitamin C has multiple actions.

Summary

Ascorbic acid is required for the formation and maintenance of normal dentin, bone, and other connective tissues of the periodontium.

Dentinogenesis in scorbutic guinea pigs fails in zones devoid of subjacent dentin with defective odontoblasts, but it appears to proceed normally over normal dentin and enamel.

Studies on scorbutic guinea pigs treated with D-ascorbic acid, 3-methylanthrene, and 2,6-dichlorophenolindolphenol indicate that each of these reagents prevent some but not all of the pathoses of scurvy. These observations suggest that vitamin C has multiple actions.

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ASCORBIC ACID AND THE PERIPHERAL VASCULAR SYSTEM*

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Introduction

Biochemical progress on the investigation of vitamin C has moved with speed since the isolation and identification of this vitamin by King and Waugh.¹ Knowledge concerning the basic physiology of its vascular manifestations, however, has failed to keep pace with the progress of chemical information. Even today we hear the phrases "capillary fragility," "decreased resistance to trauma," and "increased capillary permeability," used in the same relation to the role of vitamin C as they were used nearly 100 years ago, and with little or any more knowledge of meaning or mechanism. Because of this void, some years ago I began an evaluation of the possible role that certain nutritional factors played in the economy of the peripheral vascular system. It was natural to begin with ascorbic acid as an important substance whose role needed exploration. Concurrently, the vascular physiology of certain flavonoid materials has been evaluated.

Methods

The basic approach in my investigations has been to observe the capillary bed through the microscope while the animal is either totally anesthetized or held immobile with local anesthesia, preventing pain, and to measure the reactivity of vascular elements to certain stimuli as applied in solution or by micromanipulative methods.²⁻³ The mesentery of small or large bowel of guinea pigs or rats with suitable anesthesia was supported by a movable glass stage and bathed with constantly flowing warmed Ringer-gelatin solution (FIGURE 1). Notes were made on the presence of spontaneous vasomotor activity, the general nature of blood flow, the presence or absence of petechiae, reactivity to epinephrine in known concentrations and, in certain instances, the reactivity to stimulation with known millivolt strengths of stimuli with a suitably prepared microelectrode of silver wire.

Direct trauma was applied by single strokes with a fine camel's hair brush to vessels under direct microscopic observation. Selected arterioles, capillaries, and venules were transected with a tiny scalpel affixed to the arm of the micromanipulator. Bleeding time of such severed vessels was measured with a fast stop watch from the time of bleeding initiated by the incision to cessation of bleeding by plugging of the severed ends with platelets. Intravascular thrombosis was induced by stimulation of the vessel wall in arterioles and venules with known microvolt stimuli, and the strength of stimulation necessary to produce such thrombosis was recorded. In certain studies on rats, increased feeding of the peripheral vessels was induced by feeding Dicumarol (50 g./100 gm. of body weight) to rats at 24 hours prior to observation.

* The work reported in this paper was supported in part by the Nutrition Foundation, Inc., New York, N.Y., and the A. H. Robins Company, Richmond, Va.

The guinea pigs were on three different diets: (1) a modified Sherman-type diet; (2) a diet comparable to that used by Reid and Briggs;⁴ (3) a standard guinea pig chow laboratory diet with 15 mg. supplements of vitamin C per 100 gm. of body weight.

Animals given hesperidin or rutin were fed 10 mg./100 gm. of body weight for an average period of 16 days prior to observation. It was found that on the various vitamin C-deficiency diets, the blood level of vitamin C in the guinea pigs fell to zero in about 14 or 15 days.³

In certain experiments relating to the ability to withstand hemorrhagic shock, control guinea pigs and animals, having been on the deficiency diet for



FIGURE 1. Micromanipulative apparatus for studying small vessels in laboratory animals.

15 days, were bled according to a standard procedure.⁵ The relationship between blood loss and the duration of hypotension at which the animal survived before exitus was measured in the deficient and in the supplemented guinea pigs.

Results

The general apparatus is shown in FIGURE 1. The animals so prepared could be observed continually under the microscope for from 2 to 3 hours, and observations were made repeatedly on vessels with regard to the parameter measured. It was found that the end points of reactivity to drugs and voltage stimulation were reproducible.

In TABLE 1 the findings with regard to the reactivity and other features of

the capillary bed are shown in the control guinea pigs and in guinea pigs after 4 to 16 days on vitamin C deficiency. It is clear that scurvy per se in the animal is featured by a loss of reactivity to epinephrine stimulation in the small arterioles, by increased spontaneous fragility of the blood vessels in the capillary bed, and—especially—the location of the petechiae is almost completely on or about the collecting venular system.

FIGURE 2 displays the relationship between the total volume of blood removed from vitamin C-deficient and vitamin C-supplemented guinea pigs and

TABLE 1

	Control animals	Scorbutic animals
Epinephrine sensitivity of larger arterioles,* 100 μ in diam.	1:500,000 (1:100,000–1:5,000,000)	1:450,000 (1:100,000–1:4,000,000)
Epinephrine sensitivity of smaller arterioles,* 75 μ in diam.	1:1,000,000 (1:300,000–1:5,000,000)	No responses ever noted using 1:100,000
Epinephrine sensitivity of precapillary region†	1:2,000,000 (1:500,000–1:35,000,000)	No response ever noted using 1:100,000
Epinephrine sensitivity of small venules,* 75 μ in diam.	1:500,000 (4 animals)	No response noted using 1:100,000 (3 animals)
Capillary external diameter (μ)	7.0–10.5	7.0–11.0
Presence of vasomotor activity in arterioles and precapillaries	++++, Usually in "closed" phase	None observed; precapillaries usually opened widely
General nature of blood flow in the arterioles, capillaries, and venules	Rapid, varying with vasomotion; vessels "tonic"	Sluggish, vessels usually dilated, especially in small collecting venules
Presence of petechiae in small venules following trauma	Three in 2 of 20 animals	Present in 11 of 23 animals; numerous

* Epinephrine concentration necessary to produce narrowing to approximately 50 per cent of internal diameter.

† Epinephrine concentration necessary to produce complete closure of the vessel at this site.

the duration of the hypotension they are able to withstand before exitus. It is evident that an avitaminosis C is associated with a prominent reduction in the ability of the animal to withstand blood loss or to survive hypotension in a manner that is encountered in the control animal.

TABLES 2, 3, and 4 outline our findings in rats with regard to: the possible influence of the flavonoids hesperidin and rutin on spontaneous petechiae (TABLE 2); the clotting tendency of intravascular blood after electrical stimulation (TABLE 3); and reactivity to epinephrine (TABLE 4). Supplementation with either of these flavonoids did not influence any of the features examined. The number of spontaneous and induced petechiae, the clotting tendency in the various vessels as measured, and the reactivity to epinephrine did not

RELATION OF BLOOD LOSS
TO DURATION OF HYPOTENSION

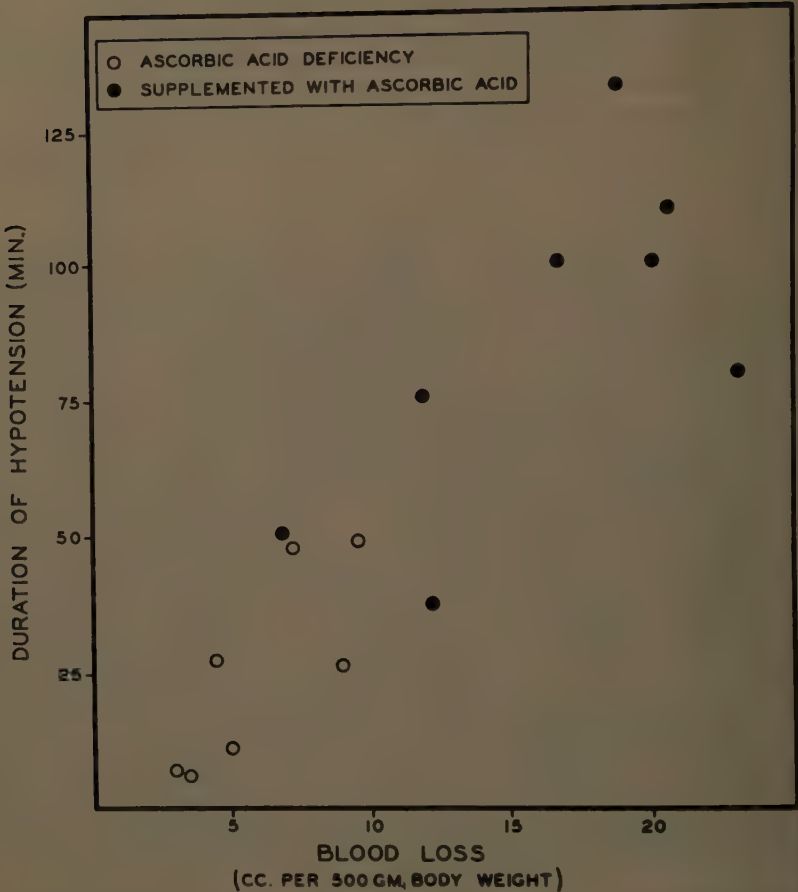


FIGURE 2. The relationship between blood loss and survival during hypotension of normal and vitamin C-deficient guinea pigs.

TABLE 2
THE APPEARANCE OF PETECHIAE IN RATS FED BIOFLAVONOIDS*

Dietary treatment	No. of animals	Spontaneous petechiae (No./field)	Fresh petechiae after brushing
Normal diet	10	7 (2-13)†	2 (0-5)†
Hesperidin-supplemented	20	9 (3-15)†	2 (0-4)†
Rutin-supplemented	20	6 (3-12)†	4 (0-6)†

* Rats received 20 to 100 mg. of dicoumarol 24 hours previous to observation.
† Numbers in parentheses indicate range of petechiae.

range. Spontaneous vasomotor activity seemed to be somewhat heightened, however, in the rutin-supplemented animals.

In TABLE 5 the bleeding time of guinea pigs on various diet classes is determined after the vessel is completely transected with a microscalpel. In general,

TABLE 3
THE EFFECT OF ELECTRICAL STIMULATION (VM)* UPON CLOTTING
TENDENCY IN THE VASCULAR BED OF THE RAT

Dietary treatment	No. of animals	Arterioles		Capillaries	Venules	
		20-50 μ	20 μ		20-50 μ	20 μ
Normal diet	9	107	70	0	82	28
Hesperidin-supplemented	20	98	87	0	63	46
Rutin-supplemented	20	110	106	0	78	34

* VM indicates volts \times milliseconds.

TABLE 4
TOPICAL APPLICATION OF EPINEPHRINE TO VESSELS OF RATS FED BIOFLAVONOIDS*

Dietary treatment	No. of animals	Epinephrine threshold	Vasomotor activity
Normal diet	9	1.6×10^{-7}	+
Hesperidin-supplemented	20	1.6×10^{-7}	++
Rutin-supplemented	20	1.6×10^{-7}	+++

* No effect was observed from application of hesperidin complex, 1 per cent, hesperidin, ethyl chalcone, homovanillic acid, dihydroxyphenylacetic acid, calcium flavonglucoside, or hydroxyphenylacetic acid.

TABLE 5
BLEEDING TIME OF GUINEA PIGS (IN SECONDS) AFTER DIRECT MICROTRANSECTION*

Dietary treatment	No. of animals	Arterioles		Capillaries	Venules	
		20-50 μ	20 μ		20-50 μ	20 μ
Normal diet	18	170	209	21	210	160
Normal diet + rutin	20	182	210	22	219	178
Normal diet + hesperidin	20	190	198	20	206	180
Vitamin C-deficient	24	206	220	23	218	174
Vitamin C-deficient + hesperidin	22	184	198	19	204	168
Vitamin C-deficient + rutin	20	197	188	22	216	157

* Data in TABLES 4, 5, and 6 are from studies on those guinea pigs given the "semisynthetic" basal diet (Reid and Briggs, 1953). Findings in approximately equal numbers of control animals fed laboratory chow alone or supplemented with rutin or hesperidin are comparable.

Vitamin C deficiency is not associated with an increase in the bleeding time, and the addition of hesperidin or rutin to normal diets or to vitamin C-deficient diets did not alter the findings in any way.

TABLE 6 illustrates the clotting tendency in guinea pigs on various types of diets. Blood clotting as induced by the microelectrode method was influenced

neither by vitamin C deficiency nor by the addition of rutin or hesperidin to either a normal or ascorbic acid-deficient diet.

TABLE 7 lists the data with regard to the effect of direct trauma (as applied with a fine camel's hair brush) to the capillary vessels of guinea pigs. The increase in hemorrhages so induced in vitamin C deficiency is apparent. This occurs in all vessel classes; as in the previous studies on spontaneous and induced petechiae, however, these ruptures are almost entirely in the venular side of the capillary bed. Rutin and hesperidin, added either to a complete

TABLE 6
CLOTING TENDENCY IN GUINEA PIGS*

Dietary treatment	No. of animals	Arterioles		Capillaries	Venules	
		20-50 μ	20 μ		20-50 μ	20 μ
Normal diet	20	55	48	0	60	35
Normal diet + rutin	30	62	60	0	54	44
Normal diet + hesperidin	28	58	66	0	72	36
Vitamin C-deficient	32	48	56	0	76	26
Vitamin C-deficient + hesperidin	24	54	62	0	54	41
Vitamin C-deficient + rutin	24	42	51	0	41	52

* Measured in volts \times milliseconds.

TABLE 7
EFFECT OF DIRECT TRAUMA TO CAPILLARY BED OF GUINEA PIGS*

Dietary treatment	No. of animals	Arterioles		Capillaries	Venules	
		20-50 μ	20 μ		20-50 μ	20 μ
Normal diet	42	0	0	0-2	0-2	0-2
Normal diet + rutin	29	0	0	0-4	0-3	0-2
Normal diet + hesperidin	40	0	0	0-3	0-2	0-4
Vitamin C-deficient	34	0	1-8	2-5	3-18	5-1
Vitamin C-deficient + hesperidin	28	0	1-9	2-6	4-16	7-1
Vitamin C-deficient + rutin	28	0	0-8	2-4	4-20	6-1

* Petechiae per low power field.

an ascorbic acid-deficient diet, did not reduce the number or the size of the petechiae.

Discussion

The foregoing data illustrates that vitamin C's role in the peripheral vascular system is one of a dynamic nature, with maintenance of peripheral vascular tone and reactivity a prime feature. The poor responsiveness to epinephrine stimulation and the moderate vasodilatation accompanying vitamin C deficiency are probably related directly to the impairment encountered in the ability of the animal to withstand hemorrhage. As blood is lost in the normal animal, the peripheral vascular system "shrinks down" by vasoconstriction, maintaining an elevated blood pressure in spite of reduced volume of circulating fluid. The reduced ability to constrict in response to stimulation can

considered a prime defect in the peripheral vascular system's role in responding to a stress situation.

The perivenular location of petechiae associated with vitamin C deficiency is a matter of interest. In this portion of the peripheral vascular bed, the smooth muscle cells about the vessel are discontinuous with relatively large areas of vessel wall consisting only of endothelium, ground substance, and collagen fibrils. With reduced tone in the smooth muscle cell, it is conceivable that the entire venular wall is weakened, the most likely site of rupture being the intermuscular cell regions; this has not yet been examined by appropriate cytological and histological methods. The dilated venule in vitamin C deficiency is probably not an exception to the concept that the dilated vessel is particularly liable to rupture solely from the distended state rather than from any mechanical structural defect in the mural elements.

The two flavonoid materials, hesperidin and rutin, examined thus far with these techniques were completely incapable of even partially relieving the disturbed physiology of avitaminosis C. The status of ascorbic acid is therefore unique in maintaining those features of the capillary bed and its component parts that have been examined with the methods outlined. With regard both to the rats and the guinea pigs, neither genus developed any symptoms on the synthetic diets that could either be explained or relieved by these two flavonoid materials. Although both hesperidin and rutin may exert certain physiological influences on the animal, it seems clear from this study that they are not essential nutrients.

Summary and Conclusions

With synthetic diets and the use of micromanipulative techniques previously developed, the role of vitamin C in peripheral vascular physiology has been examined. This substance has been found necessary to maintain responsiveness to stimulation with epinephrine, to maintain the animal's total vasocompensatory responsiveness and resistance to vascular stress, such as that produced by hemorrhage, and to maintain intact the tonic state of the venules. Hemorrhages occurring in vitamin C deficiency are primarily perivenular. With the methods used, neither rutin nor hesperidin was found to correct the vascular abnormalities of vitamin C deficiency to any degree whatever. The functional pathology of scurvy is relieved completely by ascorbic acid feedings but by neither of the two flavonoids mentioned. It is clear from these studies that the peripheral vascular role of ascorbic acid is primarily that of a "tonic," its absence being characterized by disturbed function of a widespread symptomatology of great magnitude.

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ASCORBIC ACID AND BLOOD COAGULATION*

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The hemorrhagic symptoms of scurvy have been known for centuries. In the 20th century, the isolation, synthesis, and development of quantitative methods of determination of ascorbic acid have stimulated a large number of workers to correlate the various symptoms that make up the nutritional disease, scurvy, with the *in vivo* fate of this vitamin. In spite of these studies the basic mechanisms responsible for the vascular fragility and hemorrhagic symptoms of scurvy remain unclear.

Our task is to review the possible influence of ascorbic acid on the clotting mechanism, which in turn may significantly affect a fundamental lesion of scurvy, that is, loss of vascular integrity. A recent review article, "Scurvy and Blood Coagulation,"¹ summarizes the literature in this field to date. Rather than repeat here the material covered in that report, we shall concern ourselves with the following questions: (1) May some of the hemorrhagic and coagulation phenomena seen in clinical and experimental scurvy relate to nutritional factors other than ascorbic acid? (2) What is the significance of altered sensitivity of scorbutic animals to drugs that influence coagulation? (3) What is the effect of stimulating the metabolic pathway of ascorbic acid synthesis on the clotting mechanism? (4) Can vascular integrity and coagulation factors be independently altered?

Soon after Link and his co-workers^{2,2a} isolated Dicumarol, the cause of the hypoprothrombinemia associated with hemorrhagic sweet clover disease in cattle, they undertook a study of the role of ascorbic acid in Dicumarol-induced hypoprothrombinemia.²⁻⁴ This was probably prompted by the similarity between the hemorrhagic diathesis of scurvy and that of hypoprothrombinemia. Their studies show that in certain species ascorbic acid, like vitamin K, can antagonize the action of Dicumarol and that in scurvy the hypoprothrombinemic effect of Dicumarol is enhanced. These observations are of particular interest since vitamin K deficiency itself results in frank hypoprothrombinemia while vitamin C deficiency does not.

It is known that some scorbutogenic diets supplemented with ascorbic acid do not produce perfectly normal guinea pigs. Constable⁵ has shown that the anemia seen with some scorbutogenic diets and attributed to ascorbic acid deficiency, is not seen with a more complete but still scorbutogenic diet. Could the observations of Link *et al.* be due in part to the nonspecific effects of scurvy such as starvation secondary to loss of appetite,⁶ or to the diet he employed? To test these possibilities under standard conditions we studied,⁷ in collaboration with Chenkin and Weisberg the effect of starvation on coumarin-induced hypoprothrombinemia in guinea pigs. Acenocoumarin was used in these ex-

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periments because this drug, unlike Dicumarol, could be readily dissolved at neutral pH and given intraperitoneally, thus avoiding variations in absorption. No clotting abnormality was found in acutely starved (but ascorbic acid-supplemented) guinea pigs. Paired animals, treated in addition with acenocoumarin, showed an exaggerated hypoprothrombinemic response as compared to normally fed, acenocoumarin-treated guinea pigs. There was also suggestive evidence of the presence of a circulating heparinlike clotting inhibitor in the starved group.

The possibility that drug metabolism may be slower in starved animals than in normal animals, leading to an exaggerated drug action, has been pointed out by Dixon *et al.*,⁸ who attributed this effect to changes in liver microsomes. Conney reports elsewhere in this monograph that in mild scurvy, zoxazolamine is metabolized more slowly than in control guinea pigs. In the case of acenocoumarin we did not find differences in metabolic rate in starved guinea pigs as compared with fed guinea pigs. Ninety minutes after administration of 10 mg./kg. of acenocoumarin intraperitoneally, plasma concentrations of the drug ranged from 30 to 50 mg./l. in both groups. Thus an altered rate of coumarin metabolism in starvation appears not to be the explanation for the enhanced response to coumarin in the guinea pigs.

The capacity of some drugs to stimulate biosynthesis of ascorbic acid was suggested as early as 1940.⁹ Link *et al.*,¹⁰ aware of these reports, studied the effects of vitamin C-stimulating drugs on the prothrombin response to Dicumarol. Some "vitamin C stimulators," for example, Chloretone, antagonized the action of Dicumarol in the rat. Dicumarol itself had only a transient effect on ascorbic acid excretion. It was found^{10,11} that the "stimulators" did not act in the same manner as vitamin K. However, in the rat, in spite of the effectiveness of the stimulators, large doses of ascorbic acid did not antagonize the action of Dicumarol. In contrast, as stated before, large doses of ascorbic acid did antagonize Dicumarol in the rabbit and guinea pig. Because of this apparent paradox, Link and his co-workers¹⁰ suggested that the effects of the stimulators were related to the biosynthesis of ascorbic acid rather than to ascorbic acid itself. Today there is additional evidence that this conclusion was correct.¹²

Elsewhere in this monograph are presented details of the glucuronic acid pathway of glucose metabolism and its relationship to the biosynthesis of ascorbic acid. In effect, the vitamin C stimulators are better described as "glucuronic acid pathway stimulators." Often these stimulators induce an associated increase in rate of metabolism of a variety of drugs, sometimes including the stimulator itself. Although certain species (particularly man, monkey, and guinea pig) cannot synthesize ascorbic acid from gulonic acid,¹²⁻¹⁴ these species have the ability to perform the other steps of the glucuronic acid pathway. That the pathway can be stimulated in man and guinea pig has been demonstrated by isotopic and other experiments.¹²⁻¹⁴ Thus one may expect retreatment with glucuronic acid pathway stimulators to affect the response to drugs such as the coumarins even in man and guinea pig. It was therefore of interest to us that Avellaneda¹⁵ had found that barbiturates (which are stimulators) antagonize the action of a coumarin (biscoumacetate) in man. Barbiturates themselves have no appreciable effect on coagulation.¹⁶

To test further whether glucuronic acid pathway stimulators would influence coumarin-induced hypoprothrombinemia in species unable to synthesize ascorbic acid, we chose the guinea pig¹⁷ as an experimental animal. We found that pretreatment with a stimulator (barbital) completely antagonized the action of acenocoumarin. We have also confirmed and extended similar observations in man. In human subjects the route of administration proved to be an important variable. We also investigated other known "stimulators"¹⁹, such as aminopyrine and derivatives for their ability to antagonize coumarins. Aminopyrine and particularly 4-aminoantipyrine affected the response to coumarins in quite a different manner than barbital.¹⁸ 4-Aminoantipyrine (a metabolite of aminopyrine¹⁹) induced a "hyperprothrombinemia," in untreated animals and a moderately reduced response in coumarin-treated animals. Our studies suggest that the normal guinea pig has less proconvertin activity (a component of the prothrombin complex) than other species and that this activity is increased by 4-aminoantipyrine and several other drugs. It is of interest that Link and his co-workers had also observed shortened prothrombin time after administration of caffeine and other xanthines^{2,20,21} to dogs, rats, and rabbits. We have confirmed these results. Link and his associates attributed the xanthine-induced increase in coagulability to a concomitant increase of fibrinogen. They also reported the production of hyperprothrombinemia with vitamin K²² in dogs, rats, and rabbits. We were unable to produce hyperprothrombinemia in the guinea pig⁷ with vitamin K.

Recently Soviet workers observed that large doses of ascorbic acid resulted in a transient and slight "shortened coagulation time" in humans and rabbits. Whether this is related to the ascorbic acid antagonism of coumarins as noted above is not known.

Our further studies in guinea pigs had then revealed that not all glucuronic acid pathway stimulators will block the prothrombin response to coumarins. Thus while barbiturates and Chloretone were effective, 3-methylcholanthrene by the same technique did not block response to coumarins. In fact 3-methylcholanthrene (administered intraperitoneally in oil) apparently causes release of a heparinlike substance, thus accentuating the effect of the coumarin. As noted previously, 4-aminoantipyrine also acts differently from barbital since 4-aminoantipyrine actually increased prothrombin activity (that is, shortened prothrombin time) in control animals. This latter effect is more reminiscent of the xanthine effect than it is of the glucuronic acid pathway stimulators.

From the preceding it is clear that alteration in a nutritional factor, such as ascorbic acid or its biosynthetic mechanism, may influence clotting factors in many different ways. Even when altered coagulability was not found, the possibility remained that a coagulation factor change at the tissue or cellular level was influencing vascular integrity.

Clotting abnormality is not generally described as an integral part of the syndrome of scurvy. Yet there are scattered reports that ascorbic acid shortens clotting time in man and rabbit,²³ and a number of studies¹ show that vitamin C deficiency can cause changes in platelets, prothrombin complex, and thromboplastin complex.^{24,25} On the other hand, hemorrhage into the extravascular spaces of any etiology may initiate a chain of reactions involving clotting factors.

resulting in an alteration in the coagulation properties of circulating blood. This intimate relationship between the clotting mechanism and vascular integrity makes it very difficult to determine definitely whether the clotting system is a mediator of scorbutic hemorrhage.²⁶

In conclusion, the direct effects of ascorbic acid deficiency and those effects that are secondary to the deficiency or the diet limitations imposed in order to produce the deficiency must be differentiated. Reaction to certain drugs and the fate of the drugs themselves are influenced by the factors that influence the fate and concentration of ascorbic acid in the animal. Conversely, certain drugs can influence pathways leading to ascorbic acid. These effects are frequently reflected in the response of the animal to drugs that influence coagulation. The extent to which vascular integrity is related to altered coagulability remains undetermined.

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VITAMIN C IN RELATION TO COLD TEMPERATURE TOLERANCE*

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When a warm-blooded animal is exposed to a cold environment, it responds by an increase in heat production and a decrease in heat loss. When the exposure is for prolonged periods, the chronically exposed animal develops with time a greater capability to produce heat and, consequently, a greater resistance to lethal cold than the controls (FIGURE 1) not pre-exposed to cold. Enhanced capacity to produce heat is then characteristic of animals said to be acclimatized to cold.¹ The question that we have been interested in has been to find out whether vitamin C was involved in the gradual changes leading to the increased capacity for heat production.

For that purpose we have studied (1) the effect of cold temperature on the vitamin C content of tissues, and (2) the effect of vitamin C on cold tolerance.

Effect of Cold Temperatures on the Vitamin C Content of Tissues

During exposure to cold, the concentration of vitamin C increases gradually and reaches a high plateau in all the tissues studied and in the urine of rats³ that succeed in getting acclimated² (FIGURES 2 and 3). The increase described in FIGURE 2 is much smaller if the rats exposed to cold are injected daily with vitamin C. Moreover the vitamin C content of the tissues is very low (approximately 60 per cent lower) in these rats that cannot become acclimated and are about to die.²

The same increase in tissue ascorbic acid is also found when fasted rats are exposed only partially, for short periods, to severe cold ($-15^{\circ}\text{C}.$),⁴ as shown in TABLE 1.

In guinea pigs unable to synthesize vitamin C there is also, during exposure to cold, a greater retention of ascorbic acid in the tissues (liver, kidneys, and adrenals), as shown in TABLE 2. Failure to acclimate to cold is accompanied by a decrease in the ascorbic acid content of the adrenals.²

The excretion of vitamin C,³ although increased during the first days of exposure to cold, is then decreased below the level found at room temperature; the excretion remains at a low level during the whole period of exposure to cold and even for a while when the animals are returned to room temperature (FIGURE 4).

Therefore, whether the animals studied synthesize vitamin C or not, they react to cold in the same manner: there is a retention of this substance only when acclimation to cold is successful.

Effect of Vitamin C on Cold Tolerance

In another series of experiments we were able to show that vitamin C, in large doses, is beneficial to animals such as rats,² guinea pigs,² and monkeys⁵ when they are exposed to a cold environment.

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FIGURE 5 shows a typical result obtained in guinea pigs. The percentage of survival was also always enhanced by vitamin C in rats and guinea pigs; in the latter, moreover, it was found that more and more vitamin C was needed as the temperature of exposure was lowered.²

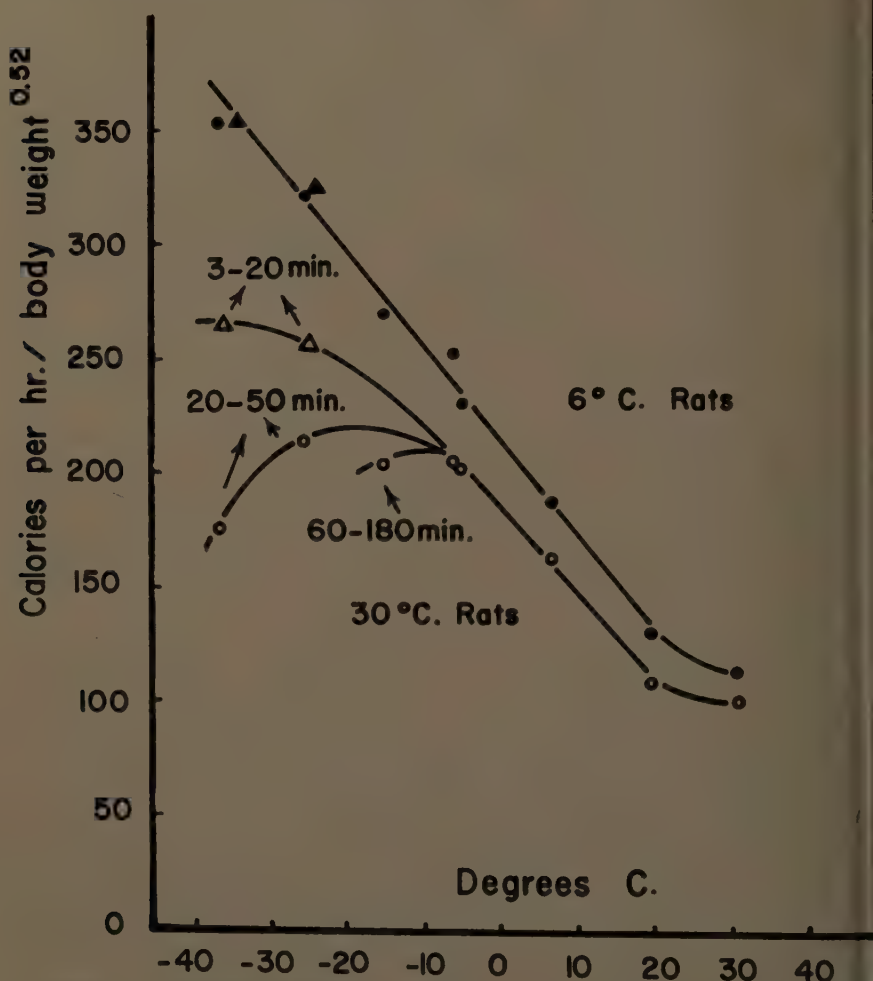


FIGURE 1. Metabolism of white rats in relation to environmental temperature, acclimation temperature (\blacktriangle , 6°C .; \triangle , 30°C .) and duration of measurement (\blacktriangle , \triangle). Test lasting from the 3rd to the 30th min. of exposure conducted with conventional open circuit apparatus. Reproduced by permission of the *Journal of Applied Physiology*.²⁴

The experiment with monkeys probably deserves that we give it further consideration in this review. Twenty-four monkeys were distributed into three groups. Group I, the control group, consisting of 11 monkeys, was kept constantly at room temperature and fed Purina Dog Chow, plus an oral supplement of 25 mg. of ascorbic acid daily for 6 months. Group II consisting of

monkeys, was given an identical diet and the same amount of ascorbic acid as the preceding group, but was maintained in a mildly cold environment of 10°C . for 6 months before any experiment was performed on it. Finally, the 5 monkeys of group III were also kept at the same mildly cold temperature for 6

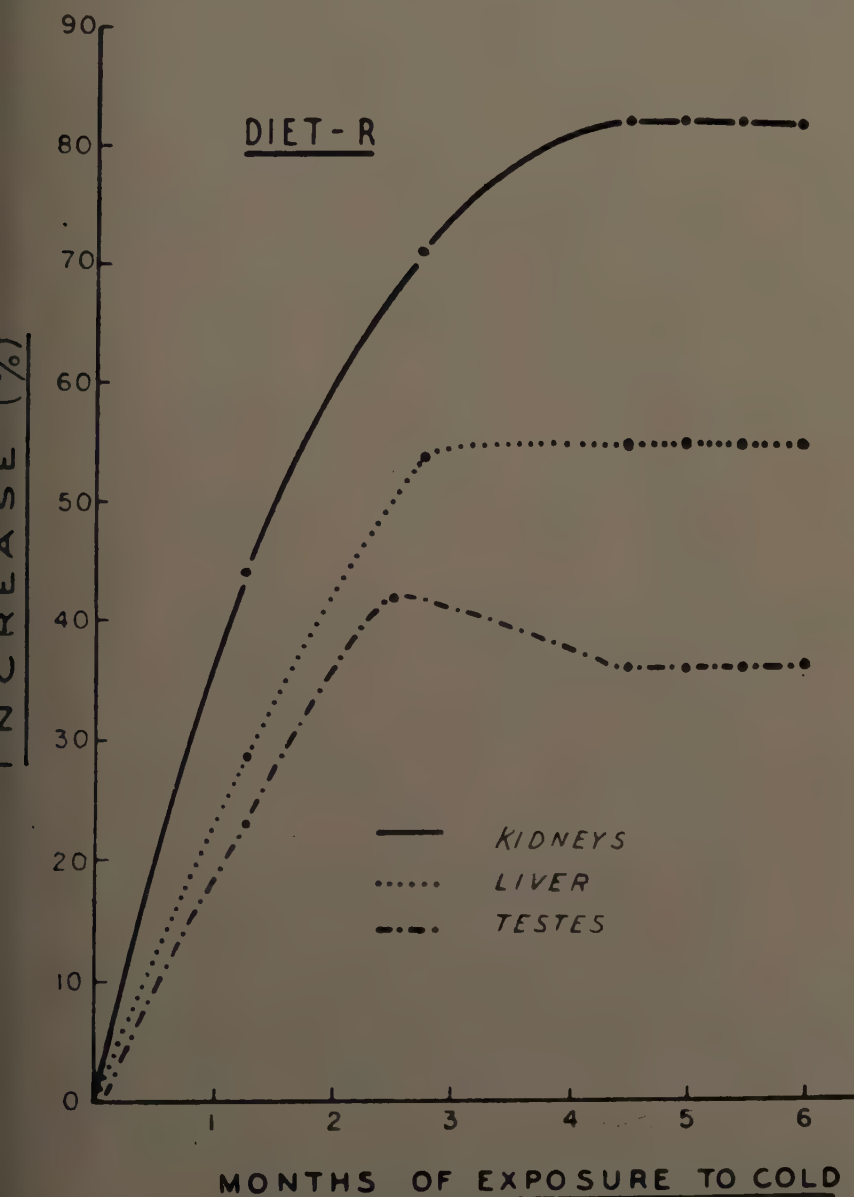
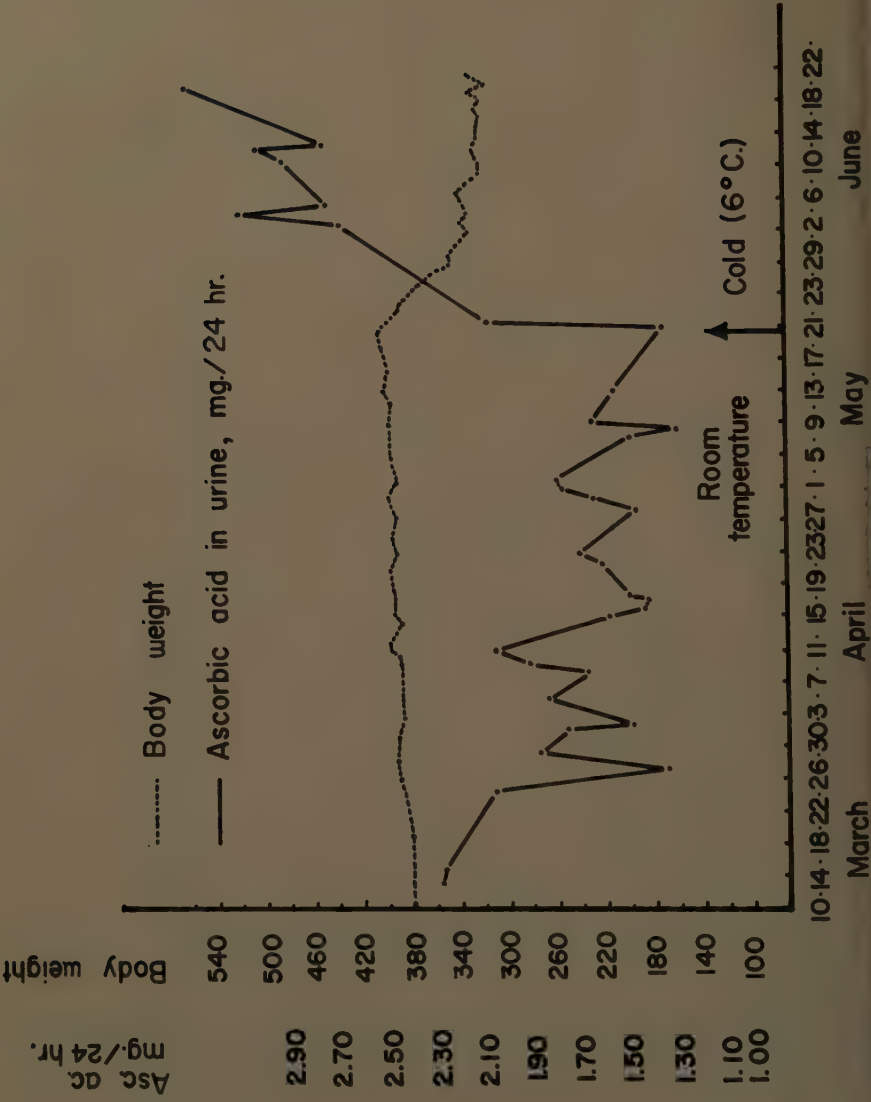


FIGURE 2. Increase in ascorbic acid concentration in tissues of rats exposed to cold as compared to the tissues of control rats kept at room temperature. Reproduced by permission of the *Canadian Journal of Research*.²



months and kept on the same diet as groups I and II, but received daily 325 g. of ascorbic acid orally. Acclimatization to cold was estimated by comparing the respective capacities of groups II and III, to resist an acute exposure of 2 hours at $-20^{\circ}\text{C}.$, with the resistance of the control group. In all these experiments the monkeys were completely at rest and immobilized during the whole exposure. The degree of resistance to that intense cold was measured by the ability of the different groups to maintain their rectal and intramuscular temperatures during exposure. The incidence of frostbite of the tails in all these monkeys was analyzed.

TABLE 1
CHANGES IN TISSUE ASCORBIC ACID IN FASTED RATS AFTER PARTIAL
EXPOSURE TO LOW TEMPERATURE

Period after frostbite (hours)	Tissue	Room temperature ascorbic acid (mg./gm.)	Low temperature ascorbic acid (mg./gm.)	Increase in ascorbic acid (%)
2½	Liver	0.225	0.328	45.7 ($t = 4.5$)
24	Liver	0.225	0.268	19.1 ($t = 2.2$)
2½	Kidneys	0.137	0.195	41.9 ($t = 2.7$)
24	Kidneys	0.137	0.142	2.9 ($t = 0.3$)
2½	Adrenals	2.79	2.93	4.6 ($t = 0.3$)
24	Adrenals	2.79	3.31	18.2 ($t = 1.3$)

TABLE 2
ASCORBIC ACID CONTENT IN SOME TISSUES OF GUINEA PIGS EXPOSED TO COLD AS COMPARED
TO THE SAME TISSUES OF GUINEA PIGS KEPT AT ROOM TEMPERATURE AND RECEIVING
THE SAME DAILY AMOUNT OF ASCORBIC ACID*

Ascorbic acid given daily (mg.)	Days of exposure to cold	Average weight during exposure (gm.)		Temperature of exposure (degrees C.)	Average ascorbic acid content (mg./gm.)		
		Initial	Final		Liver	Kidneys	Adrenals
10	15	436.1	379.5	0	0.096	0.041	0.550
10	0	420.4	498.0	20	0.065	0.035	0.369

* Reproduced by permission of the *Canadian Journal of Research*.²

The monkeys were immobilized in wooden boxes, their fingers and toes covered with mittens to prevent frostbite, but their tails were uncovered, as shown in FIGURE 6. One thermocouple was inserted in the rectum and the other one (needle type) in the right thigh. Both thermocouples were plugged to a connecting box in which eight additional thermocouples could be inserted. Never more than three monkeys were exposed at the same time. A push-button system connected any electrode with a compensated galvanometer always kept at room temperature, giving direct readings to 0.1 of a degree C. within a few seconds. The temperature of the cold room, the differential of which was $1.5^{\circ}\text{C}.$, was constantly given by a recording thermometer. The relative humidity was constant at 60 per cent.

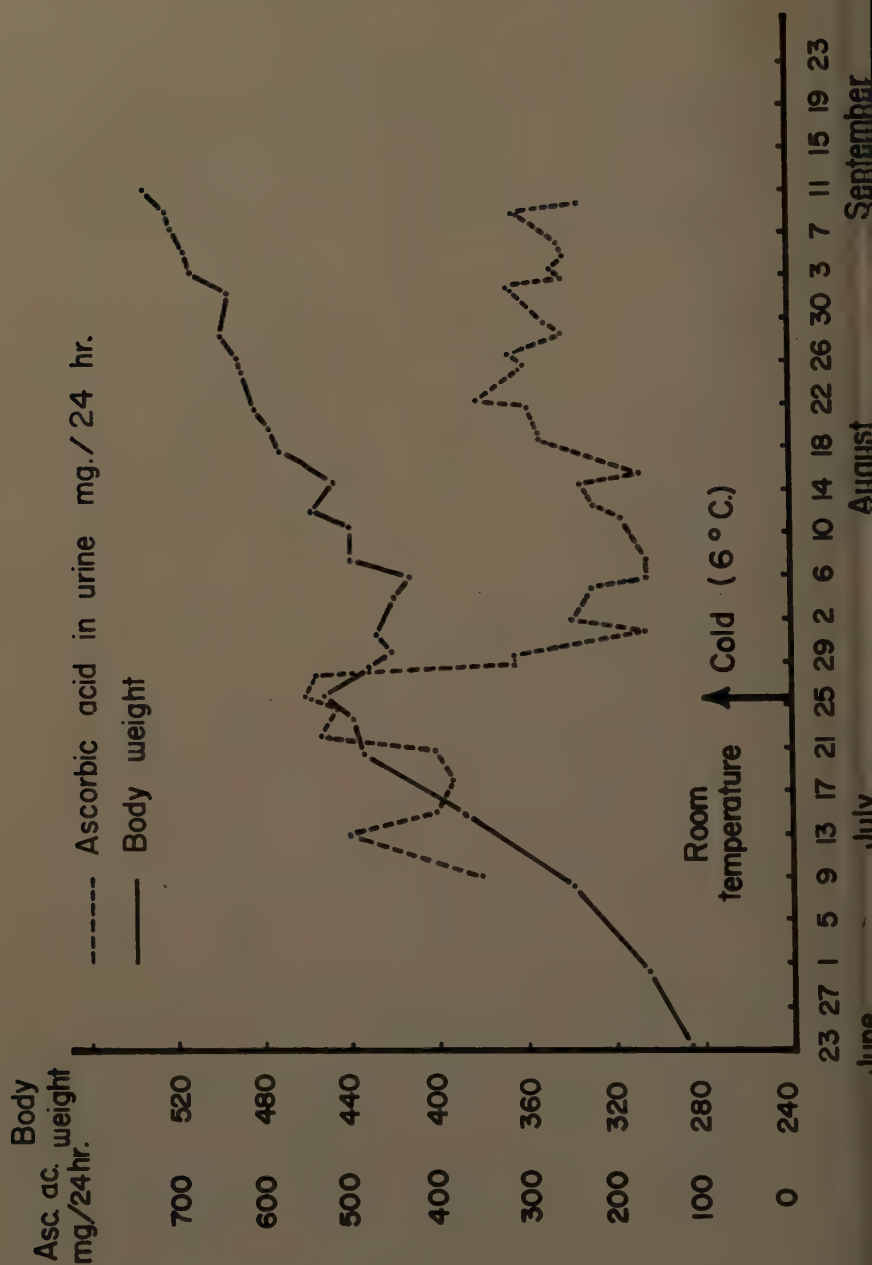


TABLE 3 presents the results obtained in the three groups of monkeys. The animals of group I had been exposed 62 times at $-20^{\circ}\text{C}.$, but never more than once a week. When they had frostbite, we waited until they were completely cured before exposing them again. The fall in rectal temperature for

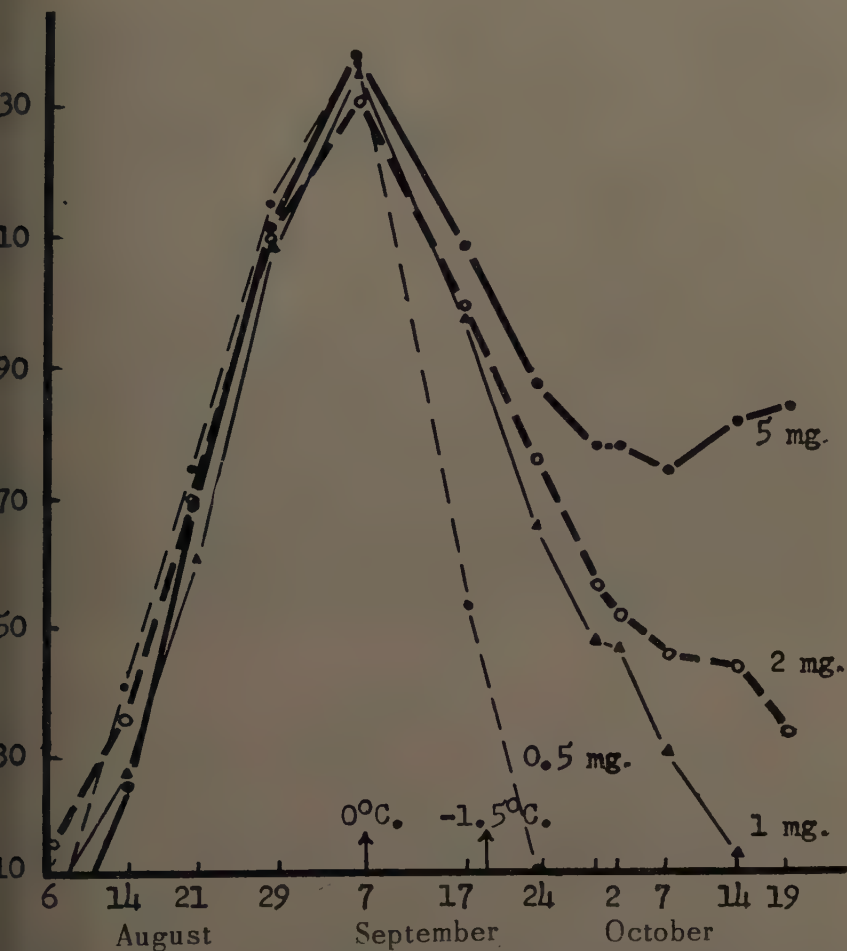


FIGURE 5. Changes in body weight due to different doses of vitamin C. Reproduced by permission of the *Canadian Journal of Research*.²

is group is $3^{\circ}\text{C}.$ Group II pre-exposed at $10^{\circ}\text{C}.$, which had grown much thicker fur, had exactly the same fall in rectal temperature. But group III, pre-exposed at the same mild cold temperature and receiving 325 mg. of ascorbic acid daily, had a fall in rectal temperature of only $2.1^{\circ}\text{C}.$, a difference of $0.9^{\circ}\text{C}.$, which is statistically very significant (the t test is 5.41 between groups I and III, and 4.74 between groups II and III). We obtain about the same results on intramuscular temperature. Group I, which had never been

exposed to cold, had a fall of 4.7° C. in intramuscular temperature when exposed to -20° C. Group II had a fall that is a little higher, but the difference between the two is not significant. Group III had a fall of only 3.6° C. Here again, the difference between group III and groups I and II is significant.



FIGURE 6.

TABLE 3
AVERAGE FALL (°C.) IN RECTAL AND INTRAMUSCULAR TEMPERATURES AT -20° C. ON EXPOSURE OF TWO HOURS*

Group	Kg.	Ascorbic acid (mg./day)	Acclimatized at °C.	No. of exposures at -20° C.	Fall in rectal temp. °C. M. and S. E.	Difference between (%)		Fall in intra-muscular temp. °C. M. and S. E.	Difference between (%)
I	5.4	25	20	62	3.07 ± 0.12	I & II 2.3	0.37	4.70 ± 0.28	I & II 6.9
II	5.6	25	10	49	3.00 ± 0.15	I & III 31.5	5.41	5.05 ± 0.41	I & III 23.0
III	5.7	325	10	31	2.10 ± 0.12	II & III 30.0	4.74	3.62 ± 0.31	II & III 28.3

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TABLE 4 shows the frequency of frostbite after exposure for 2 hours at -20° of monkeys receiving different dosages of vitamin C.

Those results, showing the beneficial effects of vitamin C in cold-exposed animals have been confirmed by Mayer,⁶ Desaulniers,⁷ Nigeon-Dubreuil,⁸ Gaarenstrom *et al.*,⁹ and Booker *et al.*¹⁰ In man exposed to cold (and on restricted diet), Leblanc *et al.*¹¹ have shown that large doses (525 mg./day) of vitamin C were beneficial: for example, skin temperature was better maintained.

oot trouble was greatly decreased as compared with the low-vitamin group (25 mg./day), and the sensation of discomfort was decreased.

Possible Mechanisms Underlying the Beneficial Effects of Vitamin C

We recently observed in our laboratory¹² that both exposure to cold and vitamin C administration accelerate the metabolism of tyrosine and phenylalanine, precursors of thyroid and medullary hormones (TABLE 5). The latter have been shown to play a leading role in enabling the acclimated animals to

TABLE 4
INCIDENCE OF FROSTBITE IN THE FOUR GROUPS OF MONKEYS
EXPOSED FOR TWO HOURS AT $-20^{\circ}\text{C}.$ *

Monkeys	Not pre-exposed to cold				Pre-exposed to cold			
	25 mg./day of ascorbic acid		325 mg./day of ascorbic acid		25 mg./day of ascorbic acid		325 mg./day of ascorbic acid	
	Number of exposures	Exposures with frostbite	Number of exposures	Exposures with frostbite	Number of exposures	Exposures with frostbite	Number of exposures	Exposures with frostbite
1					6	2	7	0
2					6	2	6	0
4					9	6	5	0
5					6	1	4	0
8					3	0	6	1
9	7	2	4	0				
10	4	2			6	0		
12	3	0			7	3		
13	6	3			5	3		
14	12	3						
18	4	2	1	0				
20			5	1				
22			4	4				
23	4	4						
Total	40	16	14	5	48	17	28	1
Per cent	40		35.7		35.4		3.6	

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acquire an increased capability for heat production: in particular, the role of the thyroid has been stressed in the elevated BMR of animals chronically exposed to cold;¹³⁻¹⁷ it has also been found^{18,19} that the thyroid hormone has a pronounced influence on the calorogenic effect of adrenaline, which may be greatly reduced¹⁸ or absent²⁰ in the absence of thyroid secretion. Hsieh and Carlson²¹ have also made the important discovery that *l*-noradrenaline has a pronounced calorogenic effect in curarized cold-acclimated rats that is absent in warm acclimated ones.

It is possible then that the beneficial effects of vitamin C may be due, at least in part, to its action on the precursors of these hormones.

Another possibility has been advanced and partly demonstrated by Deslaurais,²³ whose starting point had been a former observation²² of ours that

TABLE 5

PLASMA AMINO ACIDS IN MALE RATS (FIVE ANIMALS IN EACH GROUP)
72 HOURS IN COLD ROOM ($2.0 \pm 1.0^\circ \text{C.}$)*

Treatment: 75 Mg. of Ascorbate Administered Intraperitoneally Twice a Day for 2 Weeks Before Being Sacrificed. Equal Volumes of Normal Saline Twice a Day for Controls for Same Length of Time

Amino acid	Cold		Room temperature	
	Ascorbate-treated	Saline-treated	Ascorbate-treated	Saline-treated
Phenylalanine	—	—	Traces	+
Tyrosine	—	Traces	+	++

AMINO ACIDS IN 24-HOUR URINE SAMPLES OF MALE RATS (FIVE ANIMALS IN EACH GROUP)
72 HOURS IN COLD ROOM ($0 \pm 1.0^\circ \text{C.}$)*

Treatment: 75 Mg. of Ascorbate Administered Intraperitoneally Twice a Day for 2½ Weeks Before Being Sacrificed. Controls Received Equal Volumes of Normal Saline for the Same Length of Time

Amino acid	Cold		Room temperature	
	Ascorbate-treated	Saline-treated	Ascorbate-treated	Saline-treated
Phenylalanine	—	—	—	+
Tyrosine	—	—	Traces	++

Symbols: + indicates medium ninhydrin color. ++ indicates doubled intensity of ninhydrin color. — indicates absence.

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TABLE 6

EFFECTS OF ASCORBATE ADMINISTRATION AND THYROXINE IN
THYROIDECTOMIZED RATS EXPOSED TO COLD*

Treatment	No. animals at autopsy	Growth in gm.		Survival (%)	Thymus, fresh wt. (mg.)	Adrenals, fresh wt. (mg.)	Thyroid, height of epithelium (μ)
		0-7th day (22°C.)	7-14th day (14°C.)				
Untreated, nonoperated rats	10	32.3	30.4	100	$366.7 \pm 14.22^\dagger$	$27.8 \pm 1.93^\dagger$	$12.82 \pm 0.$
Thyroidectomized							
Ascorbate (150 mg.)	3	5.0	-15.2	20	$83.4 \pm 9.17^\dagger, \S$	$32.6 \pm 1.19^\dagger$	
Thyroxine (10 $\mu\text{g.}$)	10	15.6	31.3	100	348.2 ± 17.34	29.7 ± 2.12	
Thyroxine (3 $\mu\text{g.}$)	10	18.2	3.5	55	$207.3 \pm 23.68^\dagger$	$35.9 \pm 1.32^\dagger$	$7.97 \pm 0.$ at op- tion
Thyroxine (3 $\mu\text{g.}$) + ascorbate (150 mg.)	10	19.1	21.7	91	316.2 ± 29.73	28.6 ± 2.06	

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† Standard error of the mean.

† Significantly different from untreated, nonoperated rats.

§ Significantly different from thyroxine (3 $\mu\text{g.}$)-treated animals.

ascorbic acid prevents or decreases the adrenal hypertrophy normally found in animals exposed to cold. This observation, now confirmed by DesMarais, had made us suggest at the time that the beneficial effects of vitamin C in the cold were probably mediated through the hypophysis-adrenal axis and that the hypertrophy of the adrenals was probably more a sign of exhaustion than of activity.²² However, DesMarais has been able to show that the beneficial effects of ascorbic acid administration in animals exposed to cold are mediated chiefly through the thyroid hormones.²³ It seems very possible that ascorbic acid has a conditioning effect on the peripheral action of thyroid hormones, for example, at the level of the adrenal cortex (TABLE 6).

All these possibilities, interesting as they are, do not throw much light as yet on the intimate mechanisms involved.

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NUTRITIONAL AND ENZYMATIC STUDIES ON THE MECHANISM OF STIMULATION OF ASCORBIC ACID SYNTHESIS BY DRUGS AND CAR- CINOGENIC HYDROCARBONS*

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The work of Evans *et al.*¹ has demonstrated that the enhancement of ascorbic acid excretion by drugs involves an increased rate of formation of the vitamin and of its precursors D-glucuronate and L-gulonate. Since it seemed likely that this increased metabolic activity resulted from elevated enzyme levels, this hypothesis was subjected to two types of study. First, the ability of ethionine, an inhibitor of protein synthesis, to counteract the stimulating substances was investigated.² Second, the levels of several enzymes concerned with ascorbic acid formation were determined in the livers of normal and drug-treated animals.

Ethionine Studies

Barbital and 3-methylcholanthrene, the two stimulating chemicals used in this study, were chosen because they differ greatly from each other in structure in pharmacological action, and in the duration of their effect on ascorbic acid excretion. Dietary supplements were dissolved in the milk diet (evaporated milk-water, 1:1) supplied to the test animals. The urine of male Wistar rats (280 to 380 gm.) was collected in 5 ml. of 10 per cent oxalic acid solution and analyzed for ascorbic acid by both the 2,6-dichlorophenolindophenol and the 2,4-dinitrophenylhydrazine methods.³ The results from the two methods were consistently in agreement.

The responses of control and ethionine-treated rats to three challenges of barbital are shown in FIGURE 1. In the first two tests, in which barbital was added to the diet for 1 day, the presence of both barbital and ethionine in the milk diet decreased the food intake sufficiently to make the dosage unequal between the control and ethionine-treated groups. The average barbital intake in these tests was as follows: control group, 110 and 100 mg. per animal; ethionine-treated group, 70 and 30 mg. per animal. To ensure equal dosage a third test was carried out, the drug being injected intraperitoneally (20 mg. of sodium barbital in 1 ml. of water to each animal daily) on 3 successive days. The ethionine prevented the enhancement of ascorbic acid excretion in all 3 tests.

The effect of ethionine and of ethionine plus methionine on the action of 3-methylcholanthrene was then investigated. Control experiments confirmed the report of Conney and Burns⁴ that this hydrocarbon induces a prolonged increase in ascorbic acid excretion by rats. In FIGURE 2 it is seen that dietary ethionine effectively nullifies the action of 3-methylcholanthrene. The ethionine-treated Groups C and D failed to respond to the hydrocarbon, whereas Group E, which ingested DL-methionine as well as DL-ethionine, showed great

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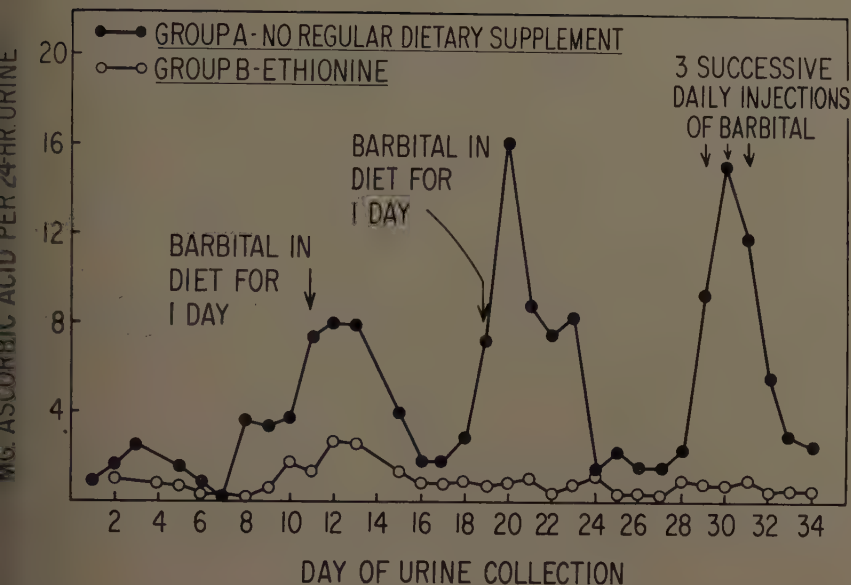


FIGURE 1. Influence of DL-ethionine on the barbitol enhancement of ascorbic acid excretion by the rat. Average values (2,6-dichlorophenolindophenol method) for the 3 animals each group. Group A received only the milk diet, whereas Group B received approximately 6 mg. of ethionine per animal per day in the milk throughout the experiment. Both groups received barbitol as indicated. In the first 2 challenges, the drug was included in the diet during days 11 and 19, respectively. In the third test, the drug was injected at the beginning of days 29, 30, and 31. See text for dosage. From Touster *et al.*²

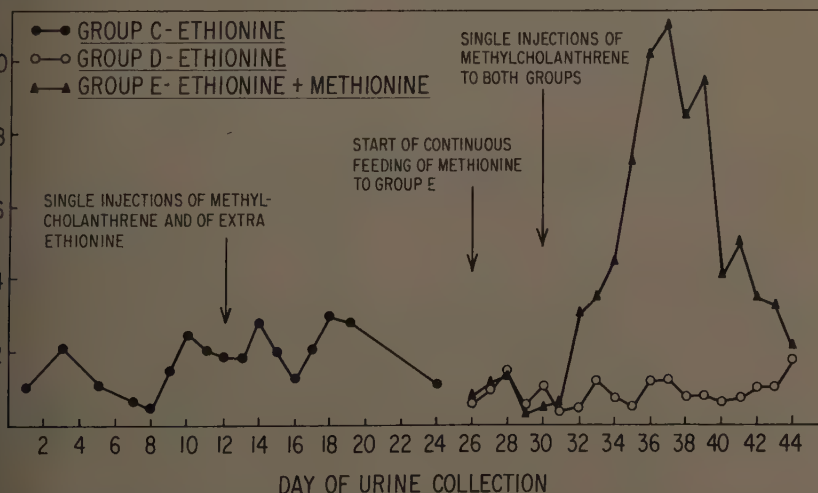


FIGURE 2. Influence of DL-ethionine and of DL-ethionine plus DL-methionine on the 3-methylcholanthrene enhancement of ascorbic acid excretion by the rat. Average values (2,6-dichlorophenolindophenol method) are given for each group. All animals ingested approximately 75 mg. of ethionine per day in the milk diet during the entire course of the experiment. At the beginning of days 12, 26, and 30, additional treatment was given as indicated on the graph. The 10 mg. of 3-methylcholanthrene (Mann) was injected intraperitoneally in 0.5 ml. of corn oil, the extra 50 mg. of ethionine on day 12 was injected in 1.0 ml. 0.9 per cent saline, and the feeding of methionine to Group E involved addition of this amino acid to the milk to give the same concentration as ethionine. Group C consisted of 6 rats. Three of these animals became Group D, 2 became Group E, and 1 was omitted in the second stage of the experiment. From Touster *et al.*²

increased urinary ascorbic acid levels. The response of Group E to methylcholanthrene was not as great or as prolonged as that of animals on milk alone but higher dosage of methionine probably would have nullified completely the ethionine block.

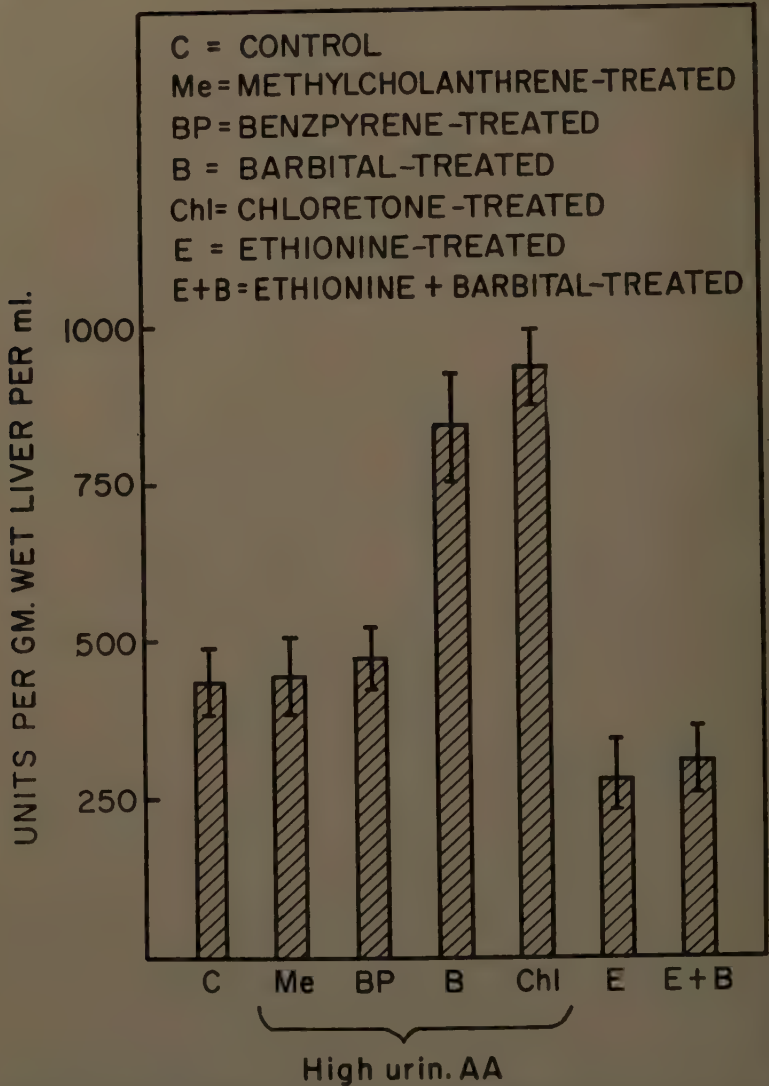


FIGURE 3. Levels of uridine diphosphate glucose dehydrogenase in the livers of normal and treated rats. See TABLE 1 for dosage and time of analysis.

Control experiments showed that, in the absence of methylcholanthrene or barbital, neither methionine nor ethionine affected appreciably the urinary ascorbic acid levels. It should be mentioned that ethionine was incorporated into the diet of ethionine-treated animals in order to saturate the animals with

the amino acid antagonist. This turned out to be a well-chosen procedure, as a later experiment showed that a single injection of 50 mg. of ethionine 30 min. before the administration of a 10-mg. dose of methylcholanthrene had only a moderate effect in diminishing the extent and duration of the enhanced ascorbic acid excretion induced by the hydrocarbon.

Enzymatic Studies

With this evidence that the enhanced excretion of ascorbic acid involves protein synthesis, presumably of an enzyme concerned with the formation of the vitamin, an attempt was made to locate the specific site affected. The isotopic studies of Evans *et al.*¹ appeared to limit the possibilities to three enzymes in the metabolic pathway leading to the formation of ascorbic acid.

TABLE 1
TREATMENT OF ANIMALS EMPLOYED IN LIVER ENZYME STUDY

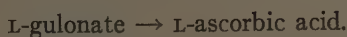
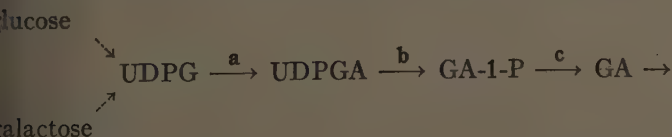
Number of animals*	Substance	Amount† and route	Day of analysis‡
5	3-Methylcholanthrene	10 mg. in 0.5 ml. corn oil I.P. on 1 day	9, 11, 15, 18, 21
2	3,4-Benzpyrene	10 mg. in 0.5 ml. corn oil I.P. for 3 days	8
4	Barbital	90 mg. in milk for 2 days	3
3	Chloretone	40 mg. in 1.0 ml. corn oil I.P. for 2 days	3
3	Ethionine	30 mg. in milk for 9 days	10
3	Barbital + ethionine	37 mg. ethionine in milk for 9 days, with 67 mg. of barbital in milk during days 8 and 9	10

* Four control animals were employed.

† Amount given is average daily dose.

‡ The day of analysis relates to the beginning of treatment of the animals in the particular group. All animals, except those given ethionine, were excreting abnormally high amounts of ascorbic acid when they were sacrificed for the liver enzyme determinations.

The fact that the synthesis of the vitamin from both glucose and galactose is readily stimulated suggests that the stimulation occurs after uridine diphosphate glucose (UDPG), the first known common intermediate derived from both hexoses; the fact that glucuronate (GA) formation is stimulated indicates that the activation is at a step preceding this intermediate:



Hence it seemed advisable to determine the levels of the enzymes catalyzing reactions a, b, and c in the livers of normal and treated animals.

Reaction a is catalyzed by UDPG dehydrogenase, a DPN-linked enzyme in the soluble fraction of liver homogenates. It was assayed by the method

of Strominger *et al.*⁵ Reaction **b** is catalyzed by a pyrophosphatase reported by Ginsberg *et al.*⁶ to be in the particulate fraction of kidney homogenates. Indirect evidence for the presence of this enzyme in liver has been obtained

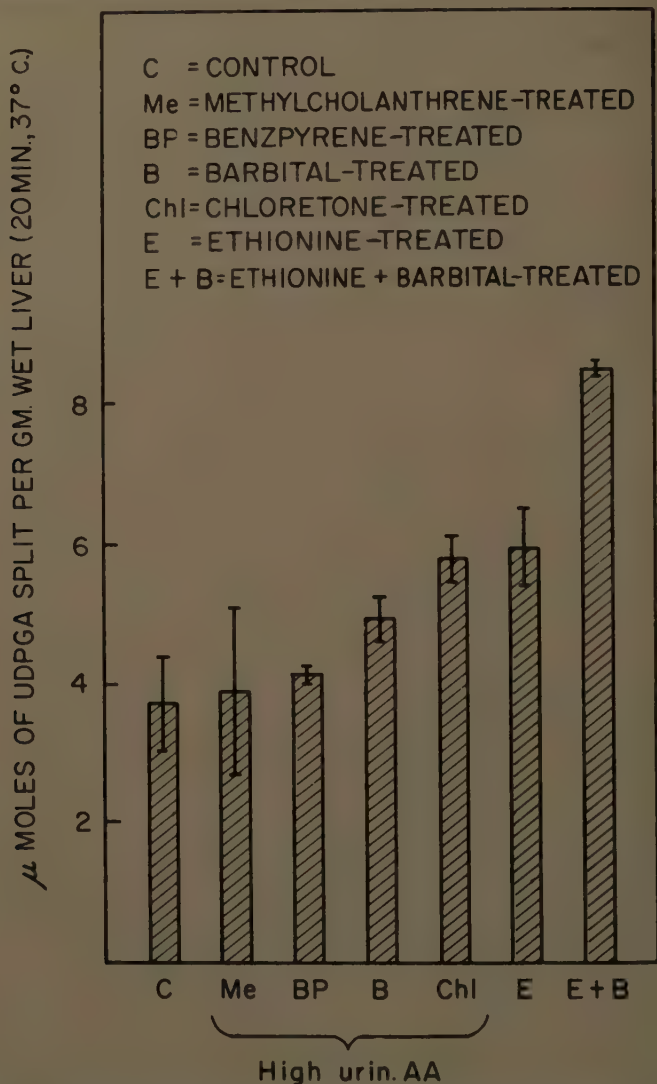


FIGURE 4. Levels of uridine diphosphate glucuronic acid pyrophosphatase in the liver of normal and treated rats. See TABLE 1 for dosage and time of analysis.

by Evans *et al.*⁷ We found that assay of liver homogenates (600 g supernate) and liver microsomes for this pyrophosphatase yielded identical results, thus demonstrating that assays could be done directly on the homogenates. The pyrophosphatase assay employed the medium of Ginsberg *et al.*⁶ with a 20 min. incubation period at 37° C., the unreacted UDPGA being assayed in the

deproteinized solution with glucuronosyl transferase.⁸ Moreover, the formation of inorganic phosphate equaled the rate of disappearance of UDPGA, suggesting that reaction **c** was rapid compared with **b**. This indicated that stimulation of **c** was less likely to be involved in the enhanced synthesis of ascorbic acid than UDPG dehydrogenase or UDPGA pyrophosphatase.

In FIGURE 3 are shown the levels of UDPG dehydrogenase in the livers of rats treated with various substances that influence ascorbic acid excretion, the dosage being given in TABLE 1. The results suggest that there may be two classes of chemicals that stimulate the glucuronate-ascorbic acid pathway. One group, the carcinogenic hydrocarbons (3-methylcholanthrene and 3,4-benzpyrene), has no effect on UDPG dehydrogenase and acts by an as yet unknown mechanism. The other group, which includes chlorbutanol (Chloretone) and barbital, induces a doubling of the level of this enzyme, an effect that might account for the increased formation of terminal substances in the pathway. In line with this hypothesis, ethionine prevents the enzyme induction by barbital when it blocks the effect of the drug on ascorbic acid formation.

The comparative study on UDPGA pyrophosphatase activity is summarized in FIGURE 4. The alterations in these enzyme levels are difficult to correlate with urinary ascorbic acid levels. At least it is evident that changes in UDPG dehydrogenase offer a more reasonable basis for interpreting the effects of the stimulating agents than do the changes in the pyrophosphatase. The increase in pyrophosphatase activity following ethionine administration is worthy of note.

It is obvious that much additional work is necessary to solve the problem under consideration. Among the questions that present themselves are the following: (1) Is the increase in UDPG dehydrogenase primarily responsible for the effects of barbital and Chloretone on ascorbic acid synthesis? (2) What mechanism is involved in the action of the carcinogenic hydrocarbons? (3) How do the various substances induce the observed changes in enzyme levels? Further experiments designed to contribute answers to these questions are now in progress.

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THIAMINE-SPARING ACTION OF ASCORBIC ACID ON SOME *LACTOBACILLI* AND THE RAT DUE TO THIAMINE DISULFIDE ACTIVATION*

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Stimulatory interaction between ascorbic acid and other water-soluble vitamins belonging to the B group has been repeatedly observed during the last two decades in conjunction with nutritional studies of both animal and microbial species. The explanation offered for such phenomena was usually twofold, as pointed out by Reid (1954) in her excellent review. Thus it was concluded generally that ascorbic acid augments the biosynthesis of some B vitamins. On the other hand, at least thiamine, pantothenic acid, and riboflavin were believed to stimulate independently the synthesis of ascorbic acid in some tissues. Consequently both views were accepted for thiamine, a factor in which I became interested recently chiefly because of the inconsistent data available on the utilization of its various forms.

Observations on the synergistic action of thiamine and ascorbic acid were made as far back as 1939 when Kasahara and his co-workers injected ascorbic acid and thiamine subcutaneously into young rats and obtained a growth enhancement. With the same levels of thiamine administered alone (0.2 μg daily thiamine per animal), the animals lost weight. On the other hand, the growth with both factors administered simultaneously matched the rich growth obtained with five-times-higher thiamine concentrations in the absence of ascorbic acid. Pigeons and guinea pigs behaved as did the rat. Independently of the Japanese authors, the Italian authors Aloisi and Polanyi (1939, 1940) found that the neurological syndromes of thiamine-deficient rats ceased immediately after administration of ascorbic acid.

Further confirmation of these findings came when Daft and Schwarz (1952) reported the prevention or delay of onset of deficiency signs in rats on thiamine free diets when 5 per cent ascorbic acid was added to the diet. This observation was made in connection with studies on the prevention of pantothenate deficiency syndromes with 2 per cent ascorbic acid.

These observations might have been considered casual and thus might have received less attention than the interaction of ascorbic acid with the other vitamins if microbiological reports had not claimed similar growth stimulation at low thiamine levels. Thus Fang and Butts (1951, 1953) described in detail the thiamine-sparing action of ascorbic acid for the growth of the heterofermentative organism *Lactobacillus fermenti* strain 36.

The authors discussed the possibilities of ascorbic acid action and rejected the idea of a simple reducing effect. Instead it was concluded that ascorbic acid participates in pyruvate metabolism as a cofactor by allowing *L. fermenti* cells to bypass the particular steps of glucose metabolism that are inhibited by

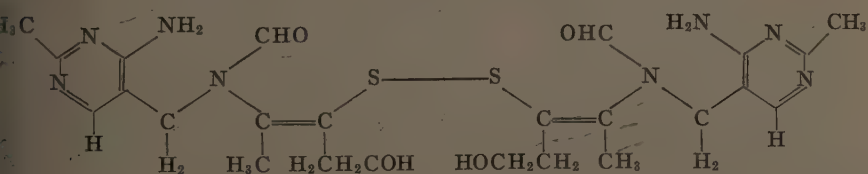
* The work on which the present survey is based was supported in part by grants from the Swedish Natural Science Research Council; from the Swedish Anticancer Society and from the Faculty of Mathematics and Natural Sciences of The University of Stockholm, all of Stockholm, Sweden.

fluoride. Although not referred to by Cavallini and his co-workers (1946), his suggestion accorded with their ideas on the direct involvement of ascorbic acid in pyruvate decarboxylation. Based on thiochrome assay of thiamine content of *L. fermenti* in the presence of either thiamine or ascorbic acid, or of both compounds, Fang and Butts (1952) also concluded that ascorbic acid is directly involved in the synthesis of thiamine by the bacterial cells.

A straightforward attempt to coordinate the microbiological and rat nutritional work on thiamine-sparing action of ascorbic acid was made recently by Terroine (1957) as a pursuit of her basic studies on nutritional vitamin interrelations (Terroine, 1955). In reference to the present discussion the accurate establishment of some peculiarities of this interrelationship are of basic importance. Thus it was shown that D-iso-ascorbic acid exerted the same protective action on both *L. fermenti* and the rat as did L-ascorbic acid, despite the lack of vitamin-C activity of the former. We should also consider Terroine's finding that the protective effect of ascorbic acid in thiamine deficiency in the rat was only of a temporary nature and that it led to a regression after about 5 to 8 weeks; moreover ascorbic acid was unable to protect the hepatic or other tissue thiamine reserves from exhaustion.

This was the state of affairs when I started work on the nutritional requirements of *L. fermenti*. My aim was by no means directed toward ascorbic acid; I was trying merely to find a more accurate microbiological estimation of thiamine. After changing the basal medium for thiamine assay with *L. fermenti* in both fluid (Bánhidi, 1958) and agar-plate test (Bánhidi, 1959), I found that the quantitative thiamine values still varied in some experiments in an unexplained and discouraging way. Much time passed before the main source of error could be identified as variations in the proportions of reduced and oxidized state of thiamine (Bánhidi, 1959a).

The reason these variations affected the growth response was traced to the nutritional unavailability of the oxidized form of thiamine disulfide:



Specific experimental conditions were required to demonstrate the full extent of inactivity of thiamine disulfide when compared with thiamine. In the basal medium, which I formulated, the alkaline-treated peptone supplement was replaced by increased amounts of acid-hydrolyzed casein and tryptophan. Additional cystine and cysteine were not added to the medium since *L. fermenti* does not need these factors for growth; nevertheless the medium contained these amino acids in small amounts that were not sufficient to interfere with the experiments. The fact that Koser and Thomas (1955) firmly established the dispensability of cystine and cysteine also for this strain of *L. fermenti* was overlooked in most of the work on thiamine assay with this organism, including work described in our own earlier papers. This is another

example of how specialization in science can sometimes render experimentation as well as interpretation of the results more complex.

After introduction of these technical changes and through aseptical addition of the thiamine and thiamine disulfide solutions to the autoclaved medium, obtained (Bánhidi, 1959a, 1960) a clear-cut answer on the inactivity of thiamine disulfide (FIGURE 1a). Activation of this compound was easily achieved

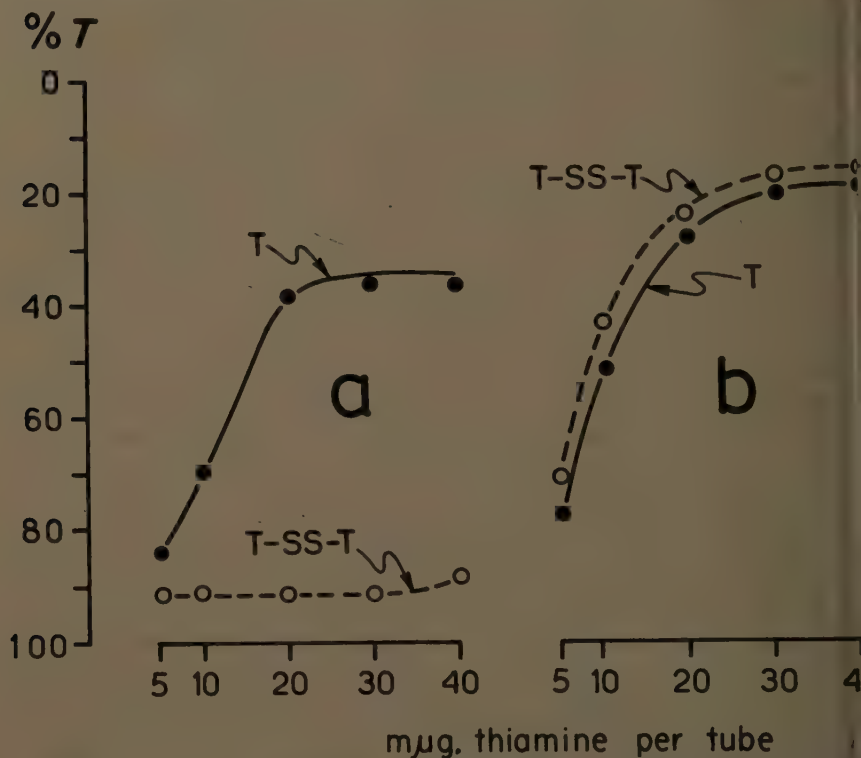


FIGURE 1. Growth of *Lactobacillus fermenti* on a chemically defined medium with thiamine (T) and thiamine disulfide (T-SS-T): (a) in the absence of cysteine and (b) with cysteine added to the medium (500 μg./ml. final volume). Turbidity readings represent mean value of duplicates or triplicates and are expressed as percentage of light transmission (% T) 5950 Å (corresponding optical density = $D = -\log T$). Reproduced by permission of the *Journal of Bacteriology*.

through the addition of suitable amounts of cysteine to the cultures (FIGURE 1b). In this way also the disulfide present in the thiamine standard solutions became activated, resulting in a typical "stimulatory" effect (cf. the curves of T and T-SS-T in FIGURES 1a and 1b).

We now know that the effect of cysteine activation of thiamine disulfide is not necessarily a simple reduction of the oxidized form to the thiazole form, since fully active thiamine-cysteine mixed disulfide might also be formed under suitable conditions (Bánhidi, 1960c). A reduction of the disulfide form by boiling, autoclaving, or through chemical agents, however, resulted in

ame activation. Thus L-ascorbic acid (FIGURES 2a and 2b) and D-iso-ascorbic acid were shown to liberate thiamine activity from thiamine disulfide, and the activity was evidently dependent on the concentrations of the ascorbic acids (FIGURES 2a and 2b) since it was the function of concentration in the case of cysteine. Naturally, similar activation could be obtained also with lower concentrations of triose reductone (von Euler, 1957), as I had reported (Bánhidi, 1960).

The next step was a nutritional trial on the rat *per analogiam* to the work of Perroine (1957) since I felt that all her results should be regarded as strong indications that ascorbic acid mobilized some scarce nutritional or endogenous inactive forms of thiamine rather than replaced thiamine by ascorbic acid in intermediary processes. In our first experiments either both forms of thiamine or only thiamine disulfide plus ascorbic acid promoted growth, depending on whether too-high or too-low molar concentrations of the thiamine derivatives were used. Finally a curative dose of 500 $\mu\text{g.}/\text{kg.}$ diet was found, which allowed us to demonstrate the necessity of activating the disulfide form of thiamine for the rat (FIGURE 3). This was shown with Wistar rats kept on a semisynthetic diet containing 68 per cent sucrose and 20 per cent vitamin-free casein after a depletion period of 2 weeks and by protection of the animal from coprophagy (Bánhidi, 1960b).

After the suitable concentration range for thiamine was established the experimental growth curves were easily reproduced, and I obtained a system in which the nutritional value of other thiamine derivatives, particularly those of mixed disulfides as well as those of some O- and S-substituted analogues, could be tested. By definition this should be done "under conditions in which thiamine disulfide requires activation for permanently curing the thiamine deficiency syndromes of the rat." FIGURE 4 (unpublished data) shows that under certain conditions in allithiamine derivative, thiamine propyl disulfide was found to be active without requiring the addition of ascorbic acid (cf., Bánhidi, 1960d).

This result was of particular interest because of the pharmacological use of the thiamine propyl disulfide and because of the inactivity of this compound toward different *Lactobacilli* requiring thiamine (Bánhidi, 1959b; 1959c; 1960a).

When raising the question of why the redox action of ascorbic acid on thiamine derivatives was more or less rejected by both Fang and Butts (1953) and Perroine (1957), one cannot escape the feeling that the reductones propagated by von Euler (1951) might deserve much wider attention in nutritional trials (Bánhidi and von Euler, 1951). This is particularly recommended in a study with ascorbic acid (Bánhidi, 1960). This retrospective question does not imply criticism of earlier work on such intricate problems. On the contrary, it should be pointed out that the inactivity of the disulfide form of thiamine in bacterial cells and higher organisms was not known at that time. It was also believed that thiamine disulfide was more active as a nutrient than the homomeric form of this vitamin (Zima *et al.*, 1953). The paradoxical fact is that this early statement might be apparently true if the batch of recrystallized thiamine disulfide were purer than the thiamine used for comparison, provided that the disulfide form was activated in that particular test through some reduction (Bánhidi, 1960). Naturally, this statement disregards the fact that

the disulfide should have been tested in its intact form in order to establish its nutritional value. The situation was even more complicated in the microbial test because of the presence of some cysteine and large amounts of cystine added to the basal medium. The latter was easily reduced by heat-sterilizing the medium.

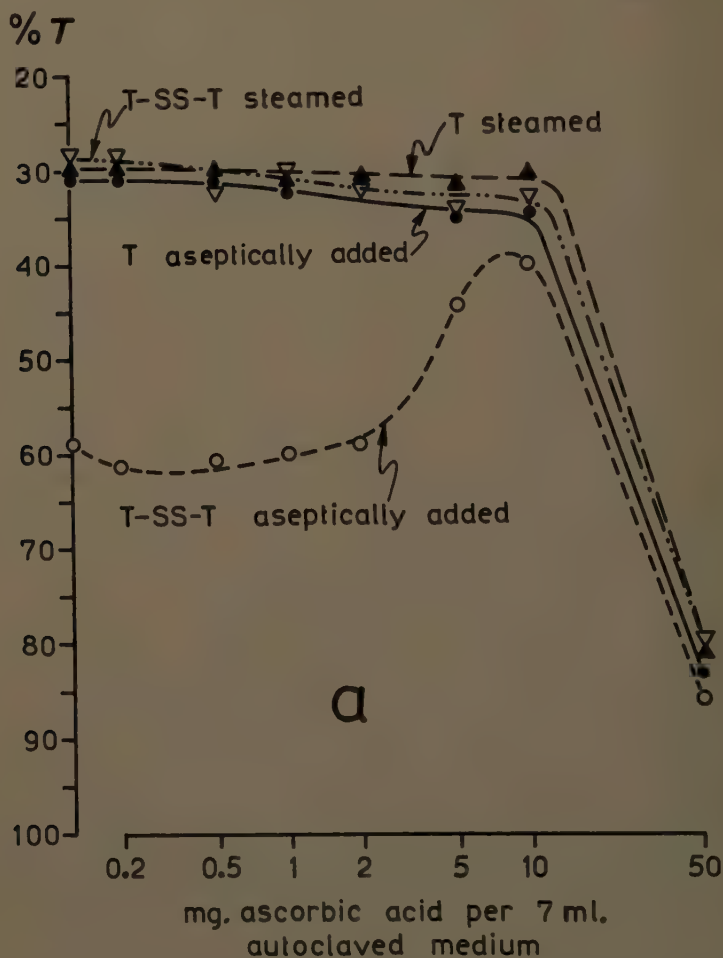


FIGURE 2. (a) Activation of thiamine disulfide (T—SS—T) to growth factor toward *Lactobacillus fermenti* by increasing concentrations of ascorbic acid in the medium. Thiamine (T) and reduced thiamine and thiamine disulfide through steaming with the medium served as controls. Reproduced by permission from the *Journal of Bacteriology*. (b), p. 329 The concentration dependence of the activation was also demonstrated with *iso*-ascorbic acid (*iso*-Asc.Ac.). Reproduced by permission from *Zeitschrift für Vitaminforschung*. In both *a* and *b* constant levels of 20 μg . thiamine (T) or 18.8 μg . thiamine disulfide (T—SS—T) were applied. The drastic fall of the curves after the highest points of activation is due to strong inhibitory action of the ascorbic acid on the bacterial growth at higher concentration. Turbidity readings represent mean values of duplicates or triplicates and expressed as percentage of light transmission (% T) at 5950 Å (corresponding optical density = $D = -\log T$).

In an early work on the nutrition of the rat, the homomeric disulfide of thiamine was found to be much less active than thiamine (Zima *et al.*, 1953). However, as I pointed out (Bánhidi, 1960*b*) at the time, no specific attention

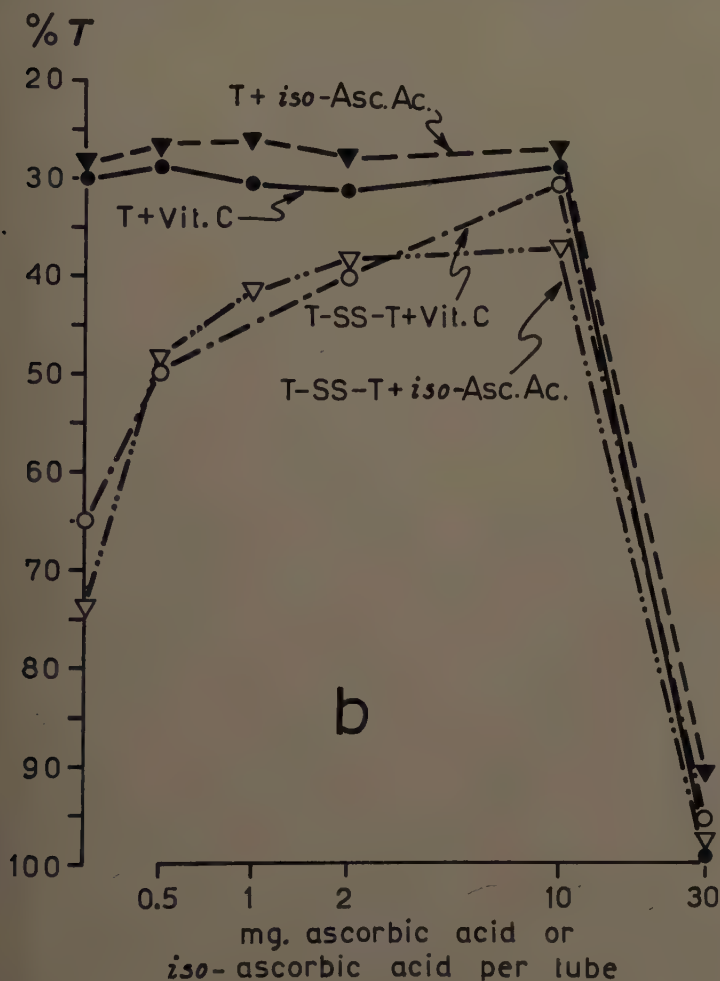


FIGURE 2.

was paid to this important fact, which stood out from a great volume of data that seemingly supported evidence contrary to this single observation.

While I believe that the disulfide activation phenomena explain some of the questions of nutritional thiamine-ascorbic-acid interaction, we must face at least two consequences: on the one hand, the results presented above may point to a useful way to study other thiamine derivatives and other B vitamins with respect to ascorbic acid synergism; on the other hand, the necessity of similar studies performed on germfree animals can hardly be overemphasized.

In one of my discussions (Bánhidi, 1960c), I stated that "convection" (nutritional features overshadowed by intestinal symbiosis) should not interfere with the demonstration of the inactivity of homomeric thiamine disulfide in the rat. This is because in my work a negative effect could be shown that pointed to the inability of the rat and its intestinal flora either to utilize or to

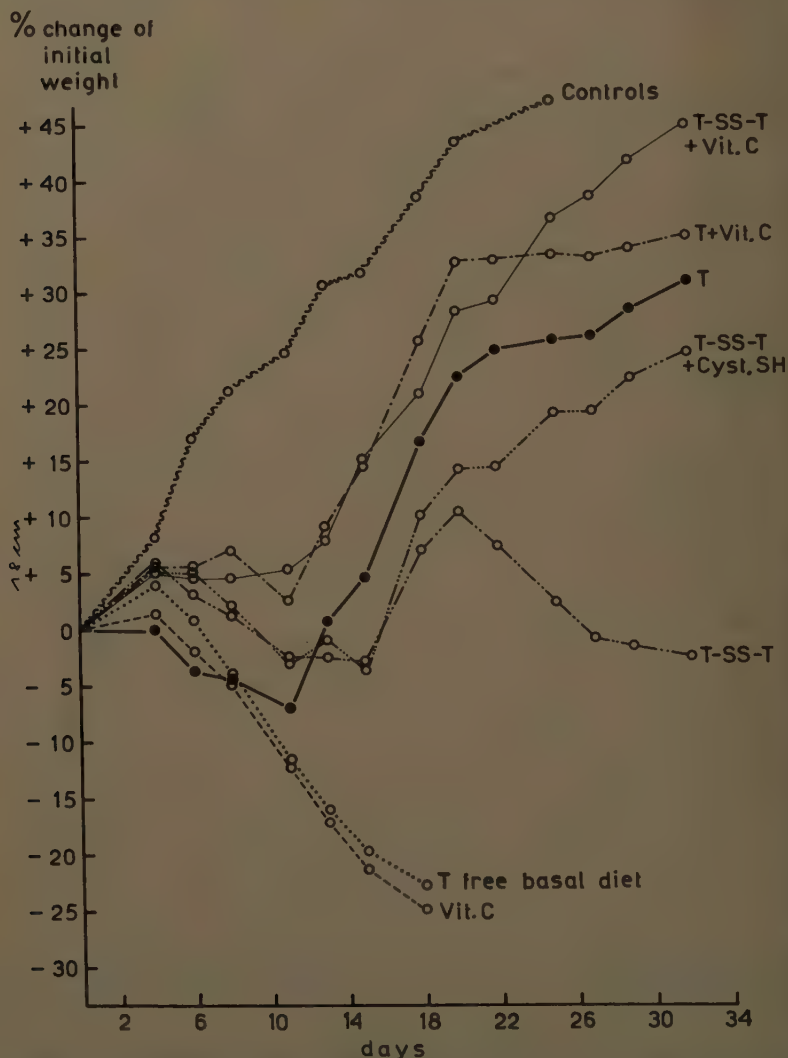


FIGURE 3. Activation of dietary thiamine disulfide (T—SS—T) by ascorbic acid (vit. C) or cysteine (Cyst.SH) for growing male albino rats (Wistar strain) as compared with growth with and without added thiamine (T) on an equimolar basis calculated as thiamine thiole. Weight gains or losses are expressed as percentage changes in total weight after 2-week total thiamine-depletion period and were plotted against time (Bánhidi, 1960b). Reproduced by permission from the *Internationale Zeitschrift für Vitaminforschung*.

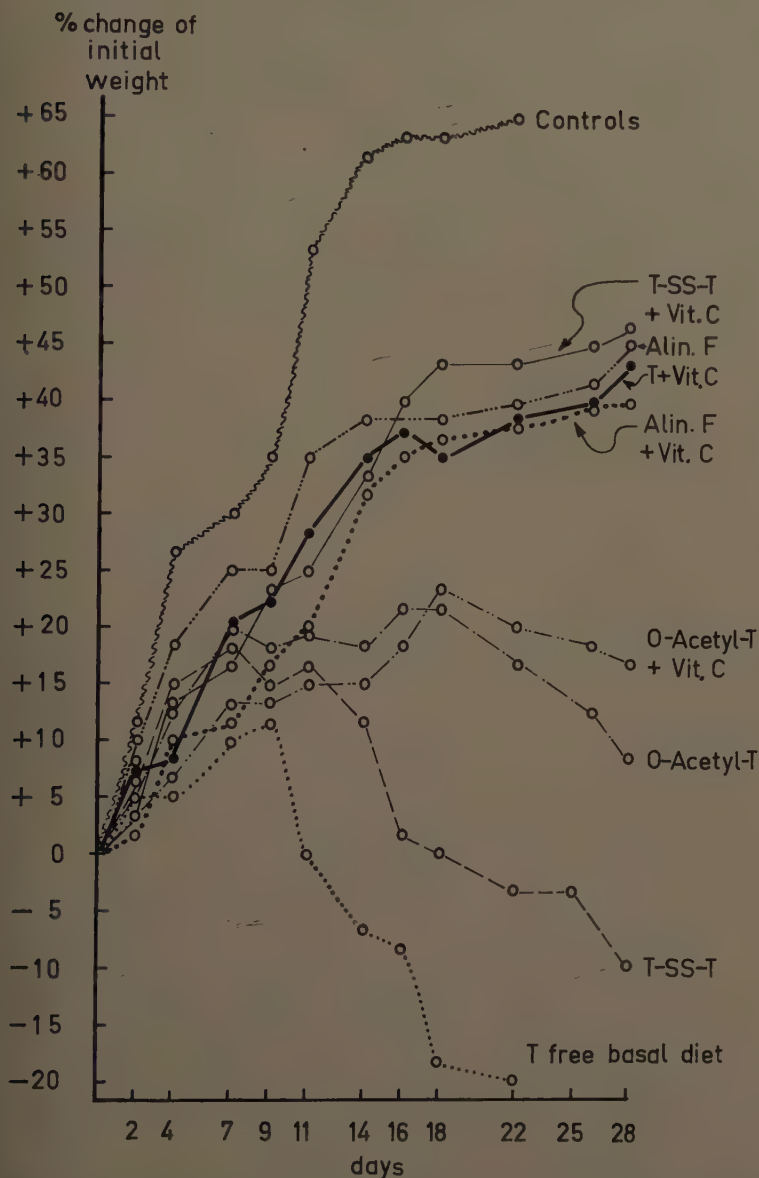


FIGURE 4. Rat growth experiment as in FIGURE 3. A mixed disulfide of the allithiamine type (thiamine propyl disulfide = "Alinamine F" of the Takeda Pharmaceutical Industries, Ltd. [Osaka, Japan] = Alin. F) was found to be active under conditions when thiamine disulfide is inactive for the rat. Purified *o*-acetyl thiamine (*o*-Acetyl-T) shows only limited activity because of the liberation of some thiamine impurities, and simultaneous addition of ascorbic acid could not result in the activation of this analogue.

reduce the disulfide form. This is, however, an exceptionally unique case, and it will be rather difficult to present similar unanimous explanations when studying active derivatives (for example, allithiamine). In these cases we must consider the action of intestinal flora as well as specific resorption conditions through the intestinal mucosa of the animals. This is indeed a field offering many problems to the nutritionist.

I certainly agree with Brown and Sturtevant (1949), who pointed out a decade ago that germfree studies are required to decide such questions as the role of intestinal synthesis of ascorbic acid—if any—for animals able to perform endogenous tissue synthesis. Concerning ascorbic acid and thiamine interaction in germfree animals, the problem of designing experiments must take into account the fact that a potential antimetabolite, oxythiamine, is formed during steam sterilization. According to Wostmann (1959) this renders thiamine one of the most vulnerable components in respect to both steam and radiation sterilization, and it represents a crucial point in germfree animal nutrition experiments.

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